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(54) Title: PSEUDOMONAS AERUGINOSA ANTIGENS

(57) Abstract: The present invention relates to proteins from *Pseudomonas aeruginosa* and their use in medicine, particularly in the preparation of vaccines and in diagnosis.

PSEUDOMONAS AERUGINOSA ANTIGENS

The present invention relates to novel antigens from *Pseudomonas aeruginosa*, and their use in medicine, particularly in the preparation of vaccines and in diagnosis.

Pseudomonas aeruginosa is a Gram negative bacteria found throughout the environment. It is non-pathogenic in healthy human beings, but is opportunistic in being able to cause disease in hosts with predisposing medical conditions. Individuals with dysfunctional lungs and immune systems, trauma patients and those with indwelling medical devices are particularly vulnerable. It is able to colonise medical devices, the lungs, the eyes and bloodstream. It accounts for up to two thirds of hospital-acquired pneumonia and is a major causative agent of Gram negative bacteremia in hospitals.

For individuals with cystic fibrosis (CF) *P. aeruginosa* is able to colonise the lungs, leading to a chronic infection that results in progressive lung damage. Infection with *P. aeruginosa* is the major cause of morbidity and mortality in cystic fibrosis patients.

Recent work suggests that colonisation of CF patients by *P. aeruginosa* can occur in early childhood, before the organism can be detected in sputum or in the absence of clinical symptoms. Once colonisation of the lungs has occurred, eradication is not possible and aggressive antibiotic therapy is the only means of controlling the infection. The etiology involves a phenotype switch to mucoid *P. aeruginosa* that is more resistant to antibiotic therapy. Once this has occurred, the clinical course of the disease is one of resistant infective episodes and poor prognosis.

Vaccines and hyperimmune globulins for *P. aeruginosa* infections have been under investigation for some thirty years. Studies in humans have investigated the use of multivalent vaccines of lipopolysaccharide, whole cell extracts, cell wall extracts, purified mixtures of toxins and antigens and conjugated vaccines. Studies on purified antigen vaccines in man and animals have focussed on the outer membrane proteins F, I and L, alginate, flagella and ribosomes. Although numerous somatic and extracellular antigens and globulins have been evaluated, with a mixture of success and failure, no treatment has gained universal acceptance. In CF patient studies, vaccination produced no benefit and there is some evidence that vaccines could accelerate disease progression by inducing an inappropriate immune response. A vaccine that is able to produce an appropriate immunotherapeutic and/or immunoprophylatic response in patients that have been colonised or susceptible to infection would be of great benefit.

WO98/32769 discloses an antigen from *P. aeruginosa* which has a molecular weight in the range of about 60 to 65 kDa and a probable N-terminal sequence of ?-E-E-K-T-P-L-T-T-A-A-?-A-P-V-V-?-N-A.

It is desirable to obtain further antigens from *P. aeruginosa* so as to allow the development of effective vaccines for *P. aeruginosa* infections for example.

Use of an animal model has allowed identification of a number of antigenic proteins isolated from *P. aeruginosa* which might represent potential vaccines suitable for the treatment and/or prevention of *P. aeruginosa* infections. The model is based on a comparison of the effectiveness of vaccine candidates with Pseudostat™, a whole-cell vaccine, the cells being inactivated, which is delivered to the small intestine. The candidate proteins were selected from whole-cell protein extracts by identifying those proteins which bound antibodies from rabbit, the rabbit having been vaccinated

subcutaneously with Pseudostat™. Those proteins recognised by this serum were tested in the animal model.

Thus in a first aspect, there is provided a protein from *P. aeruginosa* and having the following N-terminal sequence:

I A-A-K-E-(M or V)-K-F-S;

II (Q,M or V)-A-R-E-D-A-A-A-A-M;

III M-(I or L)-R-I-D-(F and/or Q); or

IV M-(L or I)-R-I-D.

In a second aspect, there is provided a protein comprising an amino acid sequence as shown in Figure 8, with the N terminal sequence of the protein being either M-I-R-I-D or Q-A-R-E-D-A-A-A-A-M.

In a third aspect, there is provided a protein comprising an amino acid sequence as shown in Figure 25 or Figure 26.

The proteins of the present invention are isolatable from *P. aeruginosa* and may be provided in substantially pure form. For example, it may be provided in a form which is substantially free of other proteins.

The invention encompasses any protein coded for by the nucleic acid sequence as shown in Figures 8, 14, 25 or 26 herein.

As discussed herein, the proteins of the invention are useful as antigenic material. Such material can be "antigenic" and/or "immunogenic". Generally, "antigenic" is taken to mean that the protein is capable of being used to raise antibodies or indeed is capable of

inducing an antibody response in a subject. "Immunogenic" is taken to mean that the protein is capable of eliciting a protective immune response in a subject. Thus, in the latter case, the protein may be capable of not only generating an antibody response but, in addition, non-antibody based immune responses.

The skilled person will appreciate that homologues or derivatives of the proteins of the invention will also find use in the context of the present invention, i.e. as antigenic/immunogenic material. Thus, for instance proteins which include one or more additions, deletions, substitutions or the like are encompassed by the present invention. In addition, it may be possible to replace one amino acid with another of similar "type". For instance, replacing one hydrophobic amino acid with another. One can use a program such as the CLUSTAL program to compare amino acid sequences. This program compares amino acid sequences and finds the optimal alignment by inserting spaces in either sequence as appropriate. It is possible to calculate amino acid identity or similarity (identity plus conservation of amino acid type) for an optimal alignment. A program like BLASTx will align the longest stretch of similar sequences and assign a value to the fit. It is thus possible to obtain a comparison where several regions of similarity are found, each having a different score. Both types of analysis are contemplated in the present invention.

In the case of homologues and derivatives, the degree of identity with a protein as described herein is less important than that the homologue or derivative should retain its antigenicity and/or immunogenicity to *P. aeruginosa*. However, suitably, homologues or derivatives having at least 60% similarity (as discussed above) with the proteins or polypeptides described herein are provided. Preferably, homologues or derivatives having at least 70% similarity, more preferably at least 80% similarity are provided. Most preferably, homologues or derivatives having at least 90%, 95%, 96, 97, 98, 99 or even 99.8% or greater similarity are provided.

In an alternative approach, the homologues or derivatives could be fusion proteins, incorporating moieties which render purification easier, for example by effectively tagging the desired protein or polypeptide. It may be necessary to remove the "tag" or it may be the case that the fusion protein itself retains sufficient antigenicity to be useful.

The invention therefore provides, in a fourth aspect, a protein which is a homologue or derivative of the proteins of the first and second aspects of the invention.

It is well known that is possible to screen an antigenic or immunogenic protein or polypeptide to identify epitopic regions, i.e. those regions which are responsible for the protein or polypeptide's antigenicity or immunogenicity. Methods well known to the skilled person can be used to test fragments and/or homologues and/or derivatives for antigenicity. For example, the fragments and/or homologues and/or derivatives can be tested to determine whether serum raised against the Pseudostat™ reacts against the fragment and/or homologue and/or derivative in question. Thus, the fragments of the present invention should include one or more such epitopic regions or be sufficiently similar to such regions to retain their antigenic/immunogenic properties. Thus, for fragments according to the present invention the degree of identity is perhaps irrelevant, since they may be 100% identical to a particular part of a protein or polypeptide, homologue or derivative as described herein. The key issue, once again, is that the fragment retains the antigenic/immunogenic properties of the protein from which it is derived.

What is important for homologues, derivatives and fragments is that they possess at least a degree of the antigenicity/immunogenicity of the protein or polypeptide from which they are derived. Thus, in a fifth additional aspect of the invention, there is

provided antigenic/or immunogenic fragments (hereinafter referred to "antigenic fragments" for convenience) of the proteins of the invention, or of homologues or derivatives thereof. In particular, the fragment may comprise the N-terminal sequence as described above.

The proteins of the present invention, or antigenic fragments thereof, can be provided alone, as a purified or isolated preparation. They may be provided as part of a mixture with one or more other *P. aeruginosa* proteins of the invention, or antigenic fragments thereof, or one or more other *P. aeruginosa* antigenic proteins or fragments thereof.

In a sixth aspect, therefore, the invention provides an antigen composition comprising one or more proteins of the invention and/or one or more antigenic fragments thereof. Such a composition can be used for the detection and/or diagnosis of *P. aeruginosa*. In one embodiment, the composition comprises one or more additional *P. aeruginosa* antigens/immunogens.

In a seventh aspect, the present invention provides a method of detecting and/or diagnosing *P. aeruginosa* which comprises:

(a) bringing into contact with a sample to be tested an antigenic protein, or an antigenic fragment thereof, or an antigen composition of the invention; and
(b) detecting the presence of antibodies to *P. aeruginosa*.

In particular, the protein, antigenic fragment thereof or antigen composition of the present invention can be used to detect IgA, IgM or IgG antibodies. Suitably, the sample to be tested will be a biological sample, e.g. a sample of blood or saliva.

In an eighth aspect, the invention provides the use of an antigenic protein, antigenic fragment thereof or an antigenic composition of the present invention in detecting and/or diagnosing *P. aeruginosa*. Preferably, the detecting and/or diagnosing is carried out *in vitro*.

The antigenic proteins, antigenic fragments thereof or antigenic composition of the present invention can be provided as a kit for use in the *in vitro* detection and/or diagnosis of *P. aeruginosa*. Thus, in a ninth aspect, the present invention provides a kit for use in the detection and/or diagnosis of *P. aeruginosa*, which kit comprises an antigenic protein, an antigenic fragment thereof or an antigenic composition of the present invention.

In addition, the antigenic protein, antigenic fragment thereof or antigen composition of the invention can be used to induce an immune response against *P. aeruginosa*. Thus, in a tenth aspect, the invention provides the use of an antigenic protein of the invention, an antigenic fragment thereof or an antigen composition of the invention in medicine.

In an eleventh aspect, the present invention provides a composition capable of eliciting an immune response in a subject, which composition comprises a protein, an antigenic fragment thereof, or an antigen composition of the invention. Suitably, the composition will be a vaccine composition, optionally comprising one or more suitable adjuvants. Such a vaccine composition may be either a prophylactic or therapeutic vaccine composition.

The vaccine compositions of the invention can include one or more adjuvants. Examples well-known in the art include inorganic gels, such as aluminium

hydroxide, and water-in-oil emulsions, such as incomplete Freund's adjuvant. Other useful adjuvants will be well known to the skilled person.

In yet further aspects, the present invention provides:

(a) the use of a protein, an antigenic fragment thereof, or an antigen composition of the invention in the preparation of an immunogenic composition, preferably a vaccine;

(b) the use of such an immunogenic composition in inducing an immune response in a subject; and

(c) a method for the treatment or prophylaxis of *P. aeruginosa* infection in a subject, or of vaccinating a subject against *P. aeruginosa* which comprises the step of administering to the subject an effective amount of a protein, at least one antigenic fragment thereof or an antigen composition of the invention, preferably as a vaccine.

In an alternative approach, the proteins described herein, or fragments thereof, can be used to raise antibodies, which in turn can be used to detect the antigens, and hence *P. aeruginosa*. Such antibodies form another aspect of the invention. Antibodies within the scope of the present invention may be monoclonal or polyclonal.

Polyclonal antibodies can be raised by stimulating their production in a suitable animal host (e.g. a mouse, rat, guinea pig, rabbit, sheep, goat or monkey) when a protein as described herein, or a homologue, derivative or fragment thereof, is injected into the animal. If desired, an adjuvant may be administered together with the protein. Well-known adjuvants include Freund's adjuvant (complete and incomplete) and aluminium

hydroxide. The antibodies can then be purified by virtue of their binding to a protein as described herein.

Monoclonal antibodies can be produced from hybridomas. These can be formed by fusing myeloma cells and spleen cells which produce the desired antibody in order to form an immortal cell line. Thus the well-known Kohler & Milstein technique (*Nature* **256** (1975)) or subsequent variations upon this technique can be used.

Techniques for producing monoclonal and polyclonal antibodies that bind to a particular polypeptide/protein are now well developed in the art. They are discussed in standard immunology textbooks, for example in Roitt *et al*, *Immunology* second edition (1989), Churchill Livingstone, London.

In addition to whole antibodies, the present invention includes derivatives thereof which are capable of binding to proteins etc as described herein. Thus the present invention includes antibody fragments and synthetic constructs, and the term "antibody" as used herein is intended to include these. Examples of antibody fragments and synthetic constructs are given by Dougall *et al* in *Tibtech* 12 372-379 (September 1994).

Antibody fragments include, for example, Fab, $F(ab')_2$ and Fv fragments. Fab fragments (These are discussed in Roitt *et al* [*supra*]). Fv fragments can be modified to produce a synthetic construct known as a single chain Fv (scFv) molecule. This includes a peptide linker covalently joining V_h and V_l regions, which contributes to the stability of the molecule. Other synthetic constructs that can be used include CDR peptides. These are synthetic peptides comprising antigen-binding determinants. Peptide mimetics may also be used. These molecules are usually conformationally restricted organic rings that mimic the structure of a CDR loop and that include antigen-interactive side chains.

Synthetic constructs include chimaeric molecules. Thus, for example, humanised (or primatised) antibodies or derivatives thereof are within the scope of the present invention. An example of a humanised antibody is an antibody having human framework regions, but rodent hypervariable regions. Ways of producing chimaeric antibodies are discussed for example by Morrison *et al* in PNAS, **81**, 6851-6855 (1984) and by Takeda *et al* in Nature. **314**, 452-454 (1985).

Synthetic constructs also include molecules comprising an additional moiety that provides the molecule with some desirable property in addition to antigen binding. For example the moiety may be a label (e.g. a fluorescent or radioactive label, or latex or an equivalent solid physical label such as an erythrocyte). Alternatively, it may be a pharmaceutically active agent.

Antibodies, or derivatives thereof, find use in detection/diagnosis of *P. aeruginosa*. Thus, in another aspect, the present invention provides a method for the detection/diagnosis of *P. aeruginosa* which comprises the step of bringing into contact a sample to be tested and antibodies capable of binding to one or more proteins of the invention, or to fragments thereof.

In addition, so-called "Affibodies" may be utilised. These are binding proteins selected from combinatorial libraries of an alpha-helical bacterial receptor domain (Nord *et al*,). Thus, small protein domains, capable of specific binding to different target proteins can be selected using combinatorial approaches.

Gene cloning techniques may be used to provide a protein of the invention in substantially pure form. These techniques are disclosed, for example, in J. Sambrook *et al Molecular Cloning* 2nd Edition, Cold Spring Harbor Laboratory Press (1989).

Thus, in a further aspect, the present invention provides a nucleic acid molecule comprising or consisting of a sequence which is:

(i) the DNA sequence set out in Figure 8, 14, 25 or 26 or its RNA equivalent;

(ii) a sequence which is complementary to the sequence of (i);

(iii) a sequence which codes for the same protein or polypeptide, as the sequence of (i) or (ii);

(iv) a sequence which is has substantial identity with any of those of (i), (ii) and (iii);

(v) a sequence which codes for a homologue, derivative or fragment of a protein as defined in Figure 8, 14, 25 or 26.

The nucleic acid molecules of the invention may include a plurality of such sequences, and/or fragments. The skilled person will appreciate that the present invention can include novel variants of those particular novel nucleic acid molecules which are exemplified herein. Such variants are encompassed by the present invention. These may occur in nature, for example because of strain variation. For example, additions, substitutions and/or deletions are included. In addition, and particularly when utilising microbial expression systems, one may wish to engineer the nucleic acid sequence by making use of known preferred codon usage in the particular organism being used for expression. Thus, synthetic or non-naturally occurring variants are also included within the scope of the invention.

The term "RNA equivalent" when used above indicates that a given RNA molecule has a sequence which is complementary to that of a given DNA molecule (allowing for the fact that in RNA "U" replaces "T" in the genetic code).

When comparing nucleic acid sequences for the purposes of determining the degree of homology or identity one can use programs such as BESTFIT and GAP (both from the Wisconsin Genetics Computer Group (GCG) software package) BESTFIT, for example, compares two sequences and produces an optimal alignment of the most similar segments. GAP enables sequences to be aligned along their whole length and finds the optimal alignment by inserting spaces in either sequence as appropriate. Suitably, in the context of the present invention compare when discussing identity of nucleic acid sequences, the comparison is made by alignment of the sequences along their whole length.

Preferably, sequences which have substantial identity have at least 50% sequence identity, desirably at least 75% sequence identity and more desirably at least 90 or at least 95% sequence identity with said sequences. In some cases, the sequence identity may be 99% or above.

Desirably, the term "substantial identity" indicates that said sequence has a greater degree of identity with any of the sequences described herein than with prior art nucleic acid sequences.

It should however be noted that, where a nucleic acid sequence of the present invention codes for at least part of a novel gene product, the present invention includes within its scope all possible sequence coding for the gene product or for a novel part thereof.

The nucleic acid molecule may be in isolated or recombinant form. It may be incorporated into a vector and the vector may be incorporated into a host. Such vectors and suitable hosts form yet further aspects of the present invention.

Therefore, for example, by using probes based upon the nucleic acid or amino acid sequences provided herein, genes in *P. aeruginosa* can be identified. They can then be excised using restriction enzymes and cloned into a vector. The vector can be introduced into a suitable host for expression.

Nucleic acid molecules of the present invention may be obtained from *P. aeruginosa* by the use of appropriate probes complementary to part of the sequences of the nucleic acid molecules. Restriction enzymes or sonication techniques can be used to obtain appropriately sized fragments for probing.

Alternatively, PCR techniques may be used to amplify a desired nucleic acid sequence. Thus the sequence data provided herein can be used to design two primers for use in PCR so that a desired sequence, including whole genes or fragments thereof, can be targeted and then amplified to a high degree. One primer will normally show a high degree of specificity for a first sequence located on one strand of a DNA molecule, and the other primer will normally show a high degree of specificity for a second sequence located on the complementary strand of the DNA sequence and being spaced from the complementary sequence to the first sequence. Typically primers will be at least 15-25 nucleotides long.

As a further alternative chemical synthesis may be used. This may be automated. Relatively short sequences may be chemically synthesised and ligated together to provide a longer sequence.

It is also possible to utilise the nucleic acid sequence shown in Figure 8, 14, 25 or 26 in the preparation of so-called DNA vaccines. Thus, the invention also provides a vaccine composition comprising one or more nucleic acid sequences as defined herein. The use of such DNA vaccines is described in the art. See for instance, Donnelly *et al*, *Ann. Rev. Immunol.*, 15:617-648 (1997).

It will also be clear that the nucleic acid sequences described herein may be used to detect/diagnose *P. aeruginosa*. Thus, in yet a further aspect, the present invention provides a method for the detection/diagnosis of *P. aeruginosa* which comprises the step of bringing into contact a sample to be tested with at least one nucleic acid sequence as described herein. Suitably, the sample is a biological sample, such as a tissue sample or a sample of blood or saliva obtained from a subject to be tested. Such samples may be pre-treated before being used in the methods of the invention. Thus, for example, a sample may be treated to extract DNA. Then, DNA probes based on the nucleic acid sequences described herein (i.e. usually fragments of such sequences) may be used to detect nucleic acid from *P. aeruginosa*.

The present invention also provides a method of vaccinating a subject against *P. aeruginosa* which comprises the step of administering to a subject a nucleic acid molecule as defined herein; a method for the prophylaxis or treatment of *P. aeruginosa* infection which comprises the step of administering to a subject a nucleic acid molecule as defined herein; and a kit for use in detecting/diagnosing *P. aeruginosa* infection comprising one or more nucleic acid molecules as defined herein.

The proteins of the present invention are potential targets for anti-microbial therapy. It is necessary, however, to determine whether each individual protein is essential for the organism's viability. Thus, the present invention also provides a method of

determining whether a protein or polypeptide as described herein represents a potential anti-microbial target which comprises inactivating said protein and determining whether *P. aeruginosa* is still viable, *in vitro* or *in vivo*.

A suitable method for inactivating the protein is to effect selected gene knockouts, i.e. prevent expression of the protein and determine whether this results in a lethal change. Suitable methods for carrying out such gene knockouts are described in Li *et al, P.N.A.S.*, **94**:13251-13256 (1997).

In a final aspect, the present invention provides the use of an agent capable of antagonising, inhibiting or otherwise interfering with the function or expression of a protein or polypeptide of the invention in the manufacture of a medicament for use in the treatment or prophylaxis of *P. aeruginosa* infection.

Preferred features of each aspect of the invention as for each other aspect, *mutatis mutandis*.

The inventors have found that the protein having N terminal sequence I has a molecular weight of about 60 kDa as determined by SDS page. Database analysis suggests that the protein is Heat Shock Protein 60 (HSP60) and has a molecular weight of 57 kDa.

Sequences II, III and IV have been identified as originating from a single protein having a molecular weight of 55-58 kDa as determined by SDS page. Database analysis suggests that the full nucleotide and amino acid sequence of this protein is as shown in Figure 8, and that the protein has a molecular weight of about 53 kDa. As is explained in more detail hereinafter, it is possible that sequences III and IV represent the N-terminal sequence of a full length protein and that sequence II

represents the N terminal sequence of the shorter protein, possibly not having a leader sequence or having been subjected to proteolysis. Database analysis suggests that the protein is a chitinase.

The invention will now be described with reference to the following examples which should not be construed as limiting the invention in any way.

The examples refer to the accompanying drawings in which:

Figure 1 shows Bioscale Q2 Anion exchange chromatography of *Pseudomonas aeruginosa* sonicate supernatant. 20mg of protein was loaded onto the column in 10mls of 20mM Tris-HCl pH 8.0. Proteins bound to the column were eluted with a 0-0.5 M NaCl gradient in 20mM Tris-HCl pH 8.0 and collected in 1 ml fractions. Fractions that were pooled were (1) 15-18, (2) 19-22, (3) 23-27, (4) 34-41.

Figure 2 shows an SDS-PAGE of Bioscale Q2 Fractions 15-27. Samples were separated on a 4-20% T polyacrylamide gel and Coomassie stained. The first two lanes are molecular weight markers, and the subsequent lanes are fractions 15-27, respectively.

Figure 3 shows SDS-PAGE of Bioscale Q2 Fractions 28-41. Samples were separated on a 4-20% T polyacrylamide gel and Coomassie stained. The first lane is molecular weight markers, and the subsequent lanes are fractions 28-41 respectively.

Figure 4 shows SDS-PAGE of Bioscale Q2 Pools 1, 2, 3 and 4. Samples were separated on a 4-20% T polyacrylamide gel and Coomassie stained. Lanes 1, 4, 7, 10 and 13 are molecular weight markers; lanes 2 and 3 are pool 1; lanes 5 and 6 are pool 2; lanes 8 and 9 are pool 3; and lanes 11 and 12 are pool 4.

Figure 5 shows a Western Blot of Bioscale Q2 Pools 1, 2, 3 and 4. Pools 1, 2, 3 and 4 were separated on a 4-20% T polyacrylamide gel and Western blotted onto a nitro-cellulose membrane. The Western blot was immunostained with Rabbit anti-Pseudostat serum, swine anti-rabbit IgG horse raddish peroxidase conjugate and developed using a 4CN substrate. Lanes 1, 4, 7, 10 and 13 are biotinylated molecular weight markers visualised with avidin peroxidase; lanes 2 and 3 are Pool 1; lanes 5 and 6 are pool 2; lanes 8 and 9 are pool 3; and lanes 11 and 12 are pool 4.

Figure 6 shows a Western blot of Bioscale Q2 Pools 1, 2, 3 and 4 For NH_2-Terminal Sequencing. Pools 1, 2, 3 and 4 were separated on a 4-20% T polyacrylamide gel and Western Blotted onto PVDF membrane. The Western blot was stained with Coomassie stain and the indicated bands were NH_2-terminal sequenced at Liverpool University. Lane 1 is molecular weight markers; lanes 2, 3 and 4 are pool 1; lanes 5, 6 and 7 are pool 2; lanes 8, 9 and 10 are pool 3; and lanes 11, 12 and 13 are pool 4.

Figure 7 shows a Western blot of Bioscale Q2 Pools 1, 2, 3 and 4 For NH_2-Terminal Sequencing. Pools 1, 2, 3 and 4 were separated on a 4-20% T polyacrylamide gel and Western Blotted onto PVDF membrane. The Western blot was stained with Coomassie stain and the indicated bands were NH_2-terminal sequenced at Liverpool University. Lane 1 is molecular weight markers; lanes 2, 3 and 4 are pool 1; lanes 5, 6 and 7 are pool 2; lanes 8, 9 and 10 are pool 3; and lanes 11, 12 and 13 are pool 4.

Figure 8 shows the DNA sequence and predicted amino acid sequence of a putative chitinase gene.

Figure 9 shows an SDS-PAGE analysis of subcellular fractions prepared from *Pseudomonas aeruginosa* isolate 385. Proteins were separated on a 4-20 % T polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lanes 1 and 2

show molecular weight standards, with sizes given in kDa. Lanes 3-10 are as follows: 3 - Growth medium after harvesting of cells; 4 - Wash solution after cell wash; 5 - TMK extract; 6 - Periplasmic proteins (cold shock extract); 7 - Supernatant from 11000 x *g* centrifugation of sonicate; 8 - Pellet from 11000 x *g* centrifugation of sonicate; 9 - Cytoplasmic proteins (supernatant from 200,000 x *g* centrifugation); 10 - Membrane proteins (pellet from 200,000 x *g* centrifugation).

Figure 10 shows an SDS-PAGE analysis of the purification of native chitinase by chitin binding. Proteins were separated on a 4-20 % T polyacrylamide gel and silver stained. Lane 1 shows molecular weight standards, with sizes given in kDa. Lanes 2-7 are as follows: 2 - Unbound *Pseudomonas aeruginosa* soluble protein extract; 3-5 - 200mM sodium phosphate buffer pH 7.0 washes 1, 2 and 3, respectively; 6 - 70 % (v/v) ethylene glycol wash of chitin; 7 - Proteins remaining bound to chitin following elution with 70 % (v/v) ethylene glycol.

Figure 11 is an immuno-stained Western blot of recombinant and native chitinases purified by hydrophobic interaction chromatography, anion-exchange chromatography and chitin binding methods. Fractions shown in Figure 20 were Western blotted, along with native and recombinant purified chitinase. Lanes 1 and 11 show biotinylated molecular weight standards, with sizes given in kDa. Lanes 2-11 are as follows: 2- HIC pooled fractions; 3-8 - Fractions 12-17, respectively, eluted from the Mono Q column; 9 - Native chitinase purified by chitin binding and eluted with 70 % (v/v) ethylene glycol; 10 - Recombinant chitinase purified by chitin binding and eluted with 80 % (v/v) ethylene glycol.

Figure 12 shows IEF of native and recombinant chitinases, purified by chitin binding. Samples were run on a Pharmacia Phast gel 3-9 with IEF standards and then silver stained. Lanes 1 and 6 show Pharmacia IEF broad range markers. Lanes

2 and 3 contain purified native chitinase and lanes 4 and 5 contain purified recombinant chitinase.

Figure 13 shows a pH activity profile for *Pseudomonas aeruginosa* isolate 385 native chitinase using 4-Nitrophenyl Diacetylchitobiose. The chitinase activity (nmoles/min) is plotted against the pH at which the assay was conducted.

Figure 14 shows the sequence of the region of the *Pseudomonas aeruginosa* isolate 385 genome that includes the chitinase (*chi*A) gene. The nucleotide sequence is translated for the coding region of the *chi*A gene, between nucleotides 161 and 1612 (inc.). The asterisk denotes a translational stop signal encoded by a TGA codon.

Figure 15 shows SDS-PAGE analysis of subcellular fractions prepared from *E. coli* JM109 cells, expressing recombinant chitinase. Proteins were separated on a 4-20 % T polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 1 shows molecular weight standards, with sizes given in kDa. Lanes 2-9 are as follows:
2 - Whole cell lysate of cells taken immediately prior to addition of IPTG to the culture; 3 - Whole cell lysate of cells taken 4 h after addition of IPTG to the culture; 4 - Periplasmic proteins extracted by cold osmotic shock; 5 - Crude sonicate of cells following osmotic shock; 6 - Supernatant from 11000 x *g* centrifugation of sonicate; 7 - Pellet from 11000 x *g* centrifugation of sonicate; 8 - Cytoplasmic proteins (supernatant from 200,000 x *g* centrifugation); 9 - Membrane proteins (pellet from 200,000 x *g* centrifugation). An arrow indicates the position to which the recombinant chitinase protein migrates.

Figure 16 shows SDS-PAGE analysis of the purification of recombinant chitinase by chitin binding. Proteins were separated on a 4-20 % T polyacrylamide gel and

stained with Coomassie Brilliant Blue R-250. Lane 1 shows molecular weight standards with sizes given in kDa. Lanes 2-8 are as follows: 2 - Unbound soluble protein extract from *E. coli* JM109 cells expressing recombinant chitinase; 3-5 - 200mM sodium phosphate buffer pH 7.0 washes 1, 2, and 3, respectively; 6-7 - 80 % (v/v) ethylene glycol washes 1 and 2, respectively; 8 - Proteins remaining bound to chitin following elution with 80 % (v/v) ethylene glycol.

Figure 17 shows a hydrophobic interaction chromatography elution profile of a soluble protein extract from *E. coli* JM109 cells expressing recombinant chitinase, loaded onto a Phenyl Sepharose High Performance column (Pharmacia XK 16/20). Elution was with a 3 M NaCl, 50 mM sodium phosphate buffer pH 6.0 to 50mM sodium phosphate buffer pH 6.0 gradient over 5 column volumes, followed by a 5 column volume wash of distilled water. 9ml fractions were collected (numbered lines). Protein elution was monitored by measurement of absorbance at 280 nm.

Figure 18 shows SDS-PAGE analysis of recombinant chitinase after hydrophobic interaction chromatography. Proteins were separated on a 4-20 % T polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 1 shows molecular weight standards, with sizes given in kDa. Lanes 2-10 are as follows: 2 - Start material before loading onto the column; 3 - Pool 1 (fractions 3, 4 and 5); 4 - Pool 2 (fractions 33-45); 5-10 - Fractions 46-51, respectively.

Figure 19 shows an anion-exchange chromatography elution profile of partially purified recombinant chitinase from hydrophobic interaction chromatography. Pooled fractions 49 and 50 from hydrophobic interaction chromatography were loaded onto a Pharmacia Mono Q column and eluted with a 0-1 M NaCl gradient in 50 mM sodium phosphate buffer pH 7.0 over 20 column volumes at a flow rate of 1

ml/min. 1 ml fractions were collected and are denoted by numbered lines. The absorbance of the eluate was continuously monitored at 280 nm.

Figure 20 shows SDS-PAGE analysis of recombinant chitinase after anion-exchange chromatography. Proteins were separated on a 4-20 % T polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 1 shows molecular weight standards, with sizes given in kDa. Lanes 2-8 are as follows: 2 - Start material before loading onto the column; 3-8 - Fractions 12-17, respectively.

Figure 21 shows an elution profile of recombinant chitinase from Superdex 200 gel filtration chromatography. 750 µl of pooled fractions purified by Mono Q was loaded onto a Superdex 200 column. Samples were eluted in 25 mM sodium phosphate buffer pH 7.0 at a flow rate of 3 ml/min and 1 ml fractions were collected. Numbered lines on the profile denoted fractions collected. Protein elution was continuously monitored by absorbance at 210 nm.

Figure 22 shows SDS-PAGE analysis of recombinant chitinase samples following each stage of purification by chromatography. Aliquots of samples at each stage of purification were analysed on a 4-20 % T polyacrylamide gel, stained with Coomassie Brilliant Blue R-250. Lane 1 shows molecular weight standards, with sizes given in kDa. Lanes 2-6 are as follows: 2 - Start material (soluble protein extract from *E. coli* JM109 cells expressing recombinant chitinase); 3 - Start material following PD10 buffer-exchange; 4 - Partially purified chitinase from hydrophobic interaction chromatography; 5 - Chitinase eluted from an anion-exchange column; 6 - Purified chitinase eluted from gel filtration column and concentrated 10 fold.

Figure 23 is an immuno-stained western blot of recombinant chitinase samples following each stage of purification by chromatography. Fractions shown in Figure

22 were Western blotted and immuno-stained with rabbit anti-*P. aeruginosa* 385 serum. Lanes 1-5 are as follows: 1 - Start material (soluble protein extract from *E.coli* JM109 cells expressing recombinant chitinase); 2 - Start material following PD10 buffer-exchange; 3 - Partially purified chitinase from hydrophobic interaction chromatography; 4 - Chitinase eluted from an anion-exchange column; 5 - Purified chitinase from gel filtration column. Lane 6 shows biotinylated molecular weight standards, with sizes given in kDa.

Figure 24 shows the pH activity profile for *Pseudomonas aeruginosa* isolate 385 recombinant chitinase using 4-Nitrophenyl Diacetylchitobiose. The chitinase activity (nmoles/min) is plotted against the pH at which the assay was conducted.

Figure 25 shows the nucleotide and amino acid sequence of the region of the *Pseudomonas aeruginosa* isolate 385 genome that includes the *gro*EL gene. The nucleotide sequence is translated for the coding region of the *gro*EL gene, between nucleotides 101 and 1744 (inc.). The asterisk denotes a translational stop signal encoded by a TAA codon.

Figure 26 shows the nucleotide sequence and deduced amino acid sequence of the *gro*EL gene fusion that is produced from the pTrcHisB expression vector.

Figure 27 shows SDS-PAGE analysis of fractions taken from extraction and purification of recombinant GroEL fusion protein. Proteins were separated on a 4-20 % T polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 1 shows molecular weight standards, with sizes given in kDa. Lanes 2-10 are as follows: 2 - B-PER soluble proteins; 3 - B-PER insoluble proteins; 4 - Proteins not bound by TALONspin column; 5 - Wash 1 of TALONspin column; 6 - Wash 2 of TALONspin column; 7 - Wash 3 of TALONspin column; 8 - Eluate from

TALONspin column; 9 and 10 - Eluate from TALONspin column after concentration. An arrow indicates the position to which the recombinant GroEL protein migrates.

Figure 28 shows an immuno-stained Western blot of fractions taken from purification of recombinant GroEL fusion protein. Fractions obtained from the purification of recombinant GroEL fusion protein were Western blotted and immuno-stained with anti- *P. aeruginosa* 385 serum. Lane 1 shows molecular weight standards, with sizes given in kDa. Lanes 2-8 are as follows: 2 - Proteins not bound by TALONspin column; 3 - Wash 1 of TALONspin column; 4 - Wash 2 of TALONspin column; 5 - Wash 3 of TALONspin column; 6 - Eluate from TALONspin column; 7 and 8 - Eluate from TALONspin column after concentration.

Example 1 - Extraction and purification of *P. aeruginosa* antigens

Culture of *P. aeruginosa*

A cryovial of *Pseudomonas aeruginosa* isolate 385 stock was removed from -80°C storage and allowed to thaw to room temperature. The organism was revived by inoculating, using a sterile transfer loop, into 5x 25 ml-glass universals, containing 10 ml of tryptone soya broth (TSB). The culture was incubated between 8-16 hours on an orbital shaker at 37°C and a speed of 200 rpm. After the initial incubation, 5% of this culture used to inoculate 4 x 500 ml culture flasks containing 250 ml of TSB. The inoculated flasks were incubated for between 16-20h on an orbital shaker at 37°C and a speed of 250 rpm, prior to harvesting.

Preparation of Whole-Cell Extract

The cultures were centrifuged in 4x 250 ml-centrifuge bottles at 5020 x g for 30 minutes on a Beckman J6 centrifuge. The supernatants were discarded and the cell pellets resuspended in 200 mls of Phosphate buffered saline (PBS), followed by centrifugation to collect the cells. This wash step was repeated twice. Following the last wash, the supernatant was removed and the cell pellets resuspended in a total of 60 mls of PBS containing 1mM 1-(2 Aminoethyl)benzenesulfonylfluoride, HCl (AEBSF protease inhibitor). *Pseudomonas aeruginosa* cells were sonicated in two 30 ml batches on a MSE Sanyo Soniprep 150 (9mm at 6 μ) using 30 seconds sonication, 60 seconds standing for 25 cycles. The samples were then centrifuged at 10000x g for 20 minutes on a Heraeus Biofuge 22R. The supernatants were pooled and filtered through a Sartorius 0.2 μm Minisart filter and stored at -40°C. A Pierce Micro BCA protein assay was performed (as per manufacturer's instructions) on the supernatant to determine the protein concentration.

Anion exchange chromatography

In order to separate *P. aeruginosa* proteins, a Bio-Rad Bioscale™ Q2 anion exchange chromatography column (7mm x 52mm) containing 2 ml of Macro-Prep Q™ was used. 20 mg of sonicate protein (1.8 ml) was diluted 10 mls in 20 mM Tris-HCl pH 8.0 (Tris buffer) and loaded onto the column. Unbound protein in the flow-through was collected and the column washed with 5 column volumes of Tris buffer. Proteins bound to the column were eluted in a 25 column volume gradient of a 0-0.5 M NaCl in 20mM Tris-HCl, pH 8.0. 1ml factions were collected throughout the gradient. A 1 M NaCl step elution was performed following the gradient to make sure no other proteins were bound. The eluate was continuously monitored by

absorbance at both 214 nm and 280 nm. The elution profile of the proteins was analysed using SDS-PAGE and Western blotting as described below.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Chromatography fractions were analysed under reducing conditions by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on precast Bio-Rad 10 % or 4-20 % T Tris-HCl gradient gels, using a Mini-PROTEAN II electrophoresis system (Bio-Rad). Samples were prepared for electrophoresis by using the following method. Bio-Rad SDS-PAGE broad range molecular weight standards were diluted 1:4 in 1x SDS-PAGE sample buffer (125mM Tris, 0.05% w/v Bromophenol Blue, 20% v/v Glycerol, 4% w/v SDS, 0.32 M DTT pH 6.8). Samples were mixed 1:1 with 2x SDS-PAGE sample buffer. Samples and standards were heated at 100°C for 4 min and 10 µl samples were loaded onto the gel. Electrophoresis was performed according to the Bio-Rad procedure at a constant 200 V until the dye front had reached the bottom of the gel (approximately 40 min). After electrophoresis gels were stained by use of 0.1% w/v Coomassie Blue R-250 in 30% v/v methanol and 10% v/v acetic acid. Proteins were visualised by destaining gels in 30% v/v methanol and 10% v/v acetic acid.

Results

Purification of *P. aeruginosa* proteins can be seen in Figure 1. 20mg of sonicate was loaded onto a Bioscale Q2 column and eluted with a 0-0.5 M NaCl gradient over 25 column volumes. Proteins were visualised using SDS PAGE and Coomassie blue staining. No proteins were detected on gels for fractions 1-14, 42-50 and 28-33, inclusive. Proteins eluted from the column can be seen on gels in Figures 2 and 3. The majority of the proteins fall into the 14-70 kDa size range, with prominent bands

present at approximately 14, 24, 42 and 50-66 kDa. Fractions were pooled for immunoblotting based on the separation of 50-66 kDa proteins identified by SDS-PAGE as follows: Pool 1, fractions 15-18; Pool 2, fractions 19-22; Pool 3, fractions 23-27; Pool 4, fractions 34-41. The pooled fractions were further analysed by SDS-PAGE (Figure 4) prior to Western blotting.

Example 2 - Western Blotting and Immunostaining

Rabbit anti-Pseudostat serum was prepared as follows. Rabbits were immunised by sub-cutaneous administration with 1 ml of 1 mg/ml of formalin-killed whole-cell *Pseudomonas aeruginosa* 385 lyophilisate (Batch: PSAE 500/01) in Freund's complete adjuvant. Booster immunizations were given on a monthly basis for 6 months and thence at 3 months intervals with 1 ml of 1 mg/ml of lyophilisate in incomplete Freund's. After 16 months the animals were killed and sera prepared. Non-immune rabbit serum was obtained from Sigma Chemical Co.

Western blotting was performed on pooled chromatography fractions. SDS-PAGE was performed as in Example 1, except that the molecular weight standards used were Bio-Rad Broad range biotinylated markers. Following SDS-PAGE, the gel was blotted for 1 hour at 15V using a Bio-Rad Trans Blot, onto a nitro-cellulose membrane, in accordance with the manufacturers' instructions. Following Western blotting, the nitro-cellulose membrane was blocked in 1% w/v bovine serum albumin (BSA) in Tris Buffered Saline (TBS) (20mM Tris-HCl, 500mM NaCl pH 7.5) for 30 minutes and than washed twice for 5 min each with Tween Tris-buffered Saline (TTBS) (TBS containing 0.05% v/v Tween 20). The blot was then incubated for 2 hours with the primary antibody, diluted in 1% w/v BSA in TTBS (1:750 dilution of Rabbit anti-Pseudostat serum). The nitro-cellulose membrane was then washed twice in TTBS (5 min each) and incubated for 1 hour with the Swine anti-Rabbit IgG horse

raddish peroxidase (HRP) conjugate (1:500 dilution) in TTBS containing 1% w/v BSA. A 1:500 dilution of Avidin Peroxidase was added to the conjugate, which binds to the Biotinylated molecular weights markers. Following incubation with the conjugate, the membrane was washed twice in TTBS and twice in TBS for 5 min each. The blot was developed by incubating with the HRP substrate 4-chloronaphthol (30 mg 4-CN in 10 ml methanol plus 50 ml TBS and 30 µl of 30% v/v H_2O_2). After a sufficient time to visualise protein antigens, the reaction was stopped by immersing the blot in deionised water for 10 minutes.

Results

The results (Figure 5) show that a number of proteins were antigenic to Rabbit anti-Pseudostat serum. The bands at approximately 6 kDa, 19 kDa and 42 kDa are at the approximate Mr of outer membrane proteins I, L and F that have previously been studied and shown to be effective vaccines in animal models. A band at ~55 kDa is present in pools 1, 2 and 3. Pools 3 and 4 contain a band at ~62 kDa.

Example 3 - NH_3-terminal sequencing

Pooled chromatography fractions were subjected to SDS PAGE and Western blotted onto a PVDF membrane. The membrane was stained with 0.025% w/v Coomassie blue R-250 dissolved in 40% v/v methanol for 10 min followed by destaining in 50% v/v methanol to visualise proteins. The membrane was dried in air at room temperature and sent for NH_2-terminal amino acid sequencing of stained protein antigens at Liverpool University. Briefly, the protein bands on the membrane were cut-out and placed in the upper cartridge of the sequencer. NH_2-terminal amino acid sequence of antigenic proteins was determined by Edman degradation using a gas

phase sequencer (Applied Biosystems), equipped with an on-line phenylthiohydantoin amino acid analyser.

Results

On two occasions, proteins in each of the pools were separated using SDS-PAGE and subsequently Western blotted onto PVDF membrane for NH_2-terminal sequencing (Figure 6 and Figure 7). The second blot was sent as a repeat to confirm protein identity. Bands 1-6 on Figure 7 were sequenced from the blot at Liverpool University. The results are shown in Table 1 below.

Table 1

Band	Sequence	Identification
1	(Q,M or V)-A-R-E-D-A-A-A-A-M	Chitinase
2	M-E-E-K-T-(P or R)-L-?-T-A	Catalase
3	A-A-K-E-(M or V)-K-F-S	HSP60
4	M-E-E-K	Catalase
5	M-(I or L)-R-I-D-(F and/or Q)	Chitinase
6	M-L or I-R-I-D	Chitinase

The likely identity of bands 2, 3 and 4 was provided by searching the NCBI databases for matching NH_2 terminal sequences in other organisms. Band 3 is likely to be a heat shock protein 60 (HSP60), and bands 2 and 4 are likely to be a catalase. The protein of bands 2 and 4 appears to be the protein of WO98/32769. The protein of band 3 does not appear to have been identified and isolated from *P. aeruginosa* before.

Searching of the NCBI databases failed to find any protein with significant homology to the sequence of bands 1, 5 and 6. Further searching was carried out as follows.

Firstly, BLAST searches were performed at the NCBI internet site (www.ncbi.nlm.nih.gov) for the sequence Q-A-R-E-D-A-A-A-A-M. A search using the BLOSUM62 matrix with default settings (Expect = 10) returned no hits. Alternative matrices are recommended for searching of short sequences. Therefore, the PAM30 matrix was used with an expect value of 100. The scores and E values obtained were relatively poor, and most matches were with internal portions of protein sequences, not with an N-terminal sequence.

A project is currently underway to obtain the genome sequence for *Pseudomonas aeruginosa* strain PA01, a common laboratory strain. The data obtained in this project is available in the project website at www.pseudomonas.com. The work is not yet complete and the data is available as unconnected contigs. This information has not yet been submitted to Genbank/EMBL databases, but is searchable at the NCBI website as unfinished genome sequences. A BLAST query of this type will search partial data for genomes of several species, all microbial, unless made more specific. The sequence Q-A-R-E-D-A-A-A-A-M was searched on the unfinished microbial genomes BLAST page at NCBI. The expect value was set to 1000 and sequence filtering was turned off (none) since it was considered that the four consecutive A's in the sequence might be discarded by the filter. In this case, the BLOSUM2 matrix was used with the TBLASTN program which converts the amino acid sequence into all possible nucleotide sequences and searches these against the databases. Only the unfinished *P. aeruginosa* genome was sequenced.

The best match had a relatively poor score and E value. However, the match was found to be 100 %. This match occurs within contig 58 of the unfinished genome

sequence (15th December 1998 release) between bases 1788 and 1759. In order to study the match further, contig 58 of the *P. aeruginosa* genome was downloaded from the www.pseudomonas.com website. Bases 1759-1788 were found to have the following sequence:

CATGGCCGCCGCGGCATCTTCGCGGGCCTG

The match obtained is with bases written as 1788-1759, which suggests that the sequence required is on the opposite strand (i.e. reverse and complement) to that shown above. Therefore the contig 58 sequence was reversed and the complement taken. Using DNASIS v2.5 software, the following bases were then searched for CAGGCCCGCGAA. This is the reverse complement of the last 12 bases shown in the sequence above. These bases were located, beginning with bases 25927 in the reverse complement of the contig 58 sequence. Translation of base 25927 to the end of this sequence showed that it encoded amino acids with the sequence beginning Q-A-R-E-D-A-A-A-A-M. This reading frame extended to a total of 473 amino acids before a STOP codon was reached. In order to determine the beginning of this open reading frame, the translation was continued back from base 25927 towards the 5' end. This revealed an ATG start codon beginning at base 25897. The codon immediately prior to this is a STOP codon, showing that the maximum extent of the open reading frame at the 5' end is the ATG mentioned above. The open reading frame concludes with a STOP codon (TGA) at bases 27346-27348 inclusive.

This open reading frame was calculated to encode a polypeptide of 53039 Da, which is very similar to the size of the protein obtained in Bands 1, 5 and 6. This provides strong evidence that the protein of band 1 is the same as that coded for by the open reading frame mentioned above.

The other hits obtained by the BLAST search were analysed in a similar way. These were all ruled out as candidates since they either did not occur within an open reading frame of a significant size or they occurred away from the N-terminus of the deduced proteins.

The protein encoded by the open reading frame was searched using the BLAST algorithm (BLOSUM62 matrix, Expect = 10) at the NCBI website. The best match was with a chitinase from *Serrata marcescens* (65% identity), and many of the other significant matches were with chitinases.

This suggests that the identified *P. aeruginosa* open reading frame encodes a chitinase. It is likely therefore that the protein of band 1 is a chitinase.

Figure 8 shows the nucleotide and predicted amino acid sequence for the putative chitinase gene. The amino acid sequence determined for band 1 corresponds to the translation of bases 31-60 inclusive. The amino acid sequence determined for bands 5 and 6 correspond respectively to the translation of bases 1-18 and 1-15 inclusive. This suggests that the inventors have obtained a sequence for both full length and processed species of the chitinase. This processing is speculated to involve removal of a signal peptide from the full length protein (bands 5 and 6) to yield the mature processed protein (band 1).

Conclusions

The examples show the fractionation of a whole cell extract from *P. aeruginosa* by anion exchange chromatography on a Bioscale Q2 column. The procedure results in the separation of a number of proteins that can be visualised by SDS-PAGE. By pooling fractions and immunoblotting with serum obtained from animals immunised

with a whole-cell inactivated *P. aeruginosa* vaccine (Pseudostat™), a number of potential protective antigens have been identified. These have been NH_2-terminally sequenced and identified through database searching as a heat shock protein (HSP60), a chitinase and a catalase. The HSP60 and chitinase represent novel antigens. Since the vaccine is known to protect against an acute lung challenge, the novel antigens represent potential vaccine candidates.

Example 4 - Purification of chitinase from *Pseudomonas aeruginosa* Isolate 385

Bacterial Strain and Growth of Cultures

Pseudomonas aeruginosa isolate 385 was obtained by culture on nutrient agar during routine microbiological analysis of sputum from a cystic fibrosis patient. Stocks of *P. aeruginosa* were stored at -80 °C in tryptone soya broth (TSB) containing 20 % (v/v) glycerol. In the laboratory, starter cultures were grown in 50 ml universal tubes by inoculating two loopfuls of stock into 25 ml of TSB and incubating for 16-20 h at 37 °C, in a shaking incubator at 250 rpm. 25 ml of starter culture was used to inoculate each of 4 x 500 ml of TSB in 1-litre shake flasks and cultures were grown at 37 °C in a shaking incubator at 250 rpm for 16-20 h.

For culture on plates, media were solidified by the incorporation of 1.5 % (w/v) agar.

Subcellular Fractionation

A small portion of the culture of *P. aeruginosa* 385 was used for subcellular fractionation to determine the distribution of the chitinase. The remainder was processed to provide material for purification of the chitinase.

Cells were harvested from cultures by centrifugation at 4000 x *g* for 2.5 h and washed with one volume of 25 mM sodium phosphate buffer pH 7.0. The wash solution was retained for analysis. Periplasmic proteins were extracted from cells obtained from 20 ml of culture by cold shock treatment, essentially as described by Hoshino (1979 ; *J. Bacteriology* **139**: 705-712). Briefly, the cells were resuspended with 1 ml TMK buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM KCl pH 7.4), then collected by centrifugation at 16000 x *g* for 4 min. The supernatant (TMK extract) was retained and the cells were resuspended with 1 ml extraction buffer (10 mM Tris-HCl, 200 mM $MgCl_2$ pH 8.4). The cells were treated by incubating at 30 °C for 5 min, followed by 15 min in an ice-water bath. This treatment was repeated and the cells were then collected by centrifugation at 16000 x *g* for 2 min and the supernatant ($MgCl_2$ extract) was retained. The cell pellet was resuspended in 1 ml Milli-Q H_2O and then incubated at room temperature for 20 min. Centrifugation was performed at 16000 x *g* for 4 min and the supernatant combined with the $MgCl_2$ extract from above. The combined cold shock extract was centrifuged twice at 16000 x *g* for 5 min, transferring the supernatant to a fresh tube after the first centrifugation, to ensure that all cells were removed. Following this extraction, the cells were resuspended in 20 ml 25 mM sodium phosphate buffer pH 7.0 containing 1 mM 1-(2-Aminoethyl)benzenesulfonylfluoride-HCl (AEBSF protease inhibitor) and disrupted by sonication using a Sanyo Soniprep 150 (19 mm probe) at an amplitude of 6 μm for 25 cycles of 30 s on and 60 s off. Cell debris and unbroken cells were removed by centrifugation at 11,000 x *g* for 30 min. The 11,000 x *g* supernatant was centrifuged further at 200,000 x *g* for 90 min to collect membranes. The resulting supernatant corresponds to cytoplasmic proteins. The membrane pellet was suspended in 1.4 ml 25 mM sodium phosphate buffer pH 7.0 by passage through a 23G hypodermic needle. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, protein content and chitinase activity were

determined for all fractions. The remaining cells from the culture (equivalent to 1980 ml of culture) were resuspended in 60 ml 25 mM sodium phosphate buffer pH 7.0 containing 1 mM AEBSF and sonicated as above in 2 x 30 ml batches. The resulting sonicate was centrifuged at 11000 x *g* for 1 h to remove cell debris and intact cells. The supernatant from this step was centrifuged further at 200,000 x *g* for 90 min. Chitinase was purified from the resulting supernatant soluble protein extract (periplasmic and cytoplasmic proteins).

Purification of Chitinase by Chitin Binding

Purification of chitinase was achieved by exploiting its strong binding to chitin. Five 100 mg aliquots of purified crab shell chitin (Sigma Co.), were mixed thoroughly in microcentrifuge tubes with 1 ml of soluble protein extract from *Pseudomonas aeruginosa* 385. The mixtures were incubated overnight at room temperature on an end over end carousel mixer. Tubes were then centrifuged at 16000 x *g* for 5 min and the supernatants removed. The chitin was washed three times with 1 ml of 200 mM sodium phosphate buffer pH 7.0 for 30 min, with centrifugation as above after each wash. The chitinase was then eluted from the chitin with 1 ml of 70 % (v/v) ethylene glycol (in 200 mM sodium phosphate buffer pH 7.0) for 30 min at room temperature and the chitin removed by centrifugation as above. The 70% (v/v) ethylene glycol supernatants were pooled and then buffer-exchanged into 25 mM sodium phosphate buffer pH 7.0 using PD10 desalting columns (see below). Recoveries and specific activity were determined by protein content and chitinase activity. The purity of the eluted chitinase was determined by SDS PAGE, isoelectric focussing (IEF) and Western blotting.

Buffer-exchange of Samples

Samples containing chitinase were buffer-exchanged by use of disposable PD10 desalting columns (Pharmacia), pre-equilibrated with 25 ml of buffer. 2.5 ml of the sample was loaded onto the column and eluted with 3.5 ml of the equilibration buffer.

Chitinase Assays

Several assays were used to determine chitinolytic activity of samples.

Chromogenic 4-Nitrophenol Chitinase Assay

4-Nitrophenyl-β-D-*N*,*N'*-Diacetylchitobiose and 4-Nitrophenyl-β-D-*N*,*N'*,*N''*-Triacetylchitotriose were prepared as stock solutions in dimethyl sulphoxide (DMSO). Chitinase activity was determined in microtitre plates. 200 µl of 200 mM sodium phosphate buffer pH 7.0 was dispensed into triplicate microwells of a microtitre plate. 25 µl of sample was then added, briefly mixed and pre-incubated for 15 min at 37 °C using the on-board peltier heated plate holder of the iEMS MF plate reader (Labsystems). The reaction was started by the addition of 25 µl of stock substrate. The absorbance at 410 nm was read every 15 seconds for a total of 15 min and the maximum initial rate of reaction calculated. The extinction coefficient for 4-nitrophenol at 410 nm was calculated by using the same assay buffer and volumes with standard 4-nitrophenol solutions in microtitre plates and used to convert rates to nmoles of substrate per min. For routine assays, to follow purification, 1 mM 4-Nitrophenyl-β-D-*N*,*N'*-Diacetylchitobiose was used. One unit was defined as the conversion of 1 nmole/min. The assay was linear with respect to initial rates and enzyme concentration. K_m and K_{cat} for 4-Nitrophenyl-β-D-*N*,*N'*-

Diacetylchitobiose and 4-Nitrophenyl-β-D-*N*,*N*',*N*''-Triacetylchitotriose were determined for the purified enzyme using the microtitre plate assay described above, but varying the substrate concentration. Stocks of 30 mM 4-Nitrophenyl-β-D-*N*,*N*'-Diacetylchitobiose and 4.45 mM 4-Nitrophenyl-β-D-*N*,*N*',*N*''-Triacetylchitotriose were prepared in DMSO and diluted to various concentrations in DMSO. The K_m and V_{max} were calculated from direct linear plots (Cornish-Bowden, A. & Eisenthal, R. 1974. Biochem. J., 139, 721) of the data.

Fluorogenic 4-Methylumbelliferone Chitinase Assay

4-Methylumbelliferyl *N*-Acetyl-β-D-Glucosaminide, 4-Methylumbelliferyl-β-D-*N*,*N*'-Diacetylchitobioside, 4-Methylumbelliferyl-β-D-*N*,*N*',*N*''-Triacetylchitotrioside and 4-Methylumbelliferyl-β-D-*N*,*N*',*N*'',*N*'''-Tetraacetylchitotetraoside were prepared as 1 mM stock solutions in DMSO.

Chitinase activity was determined in black microtitre plates. 150 µl of 200 mM sodium phosphate buffer pH 7.0 was dispensed into triplicate microwells of a microtitre plate. 20 µl of substrate was then added and briefly mixed before incubating for 15 min at 37 °C using the on board incubator of the Fluoroskan plate reader (Labsystems). The reaction was started by the addition of 30 µl of chitinase. After 15 min incubation, the reaction was stopped by the addition of 50 µl of 3 M sodium carbonate and the fluorescence caused by the release of 4-methylumbelliferone determined at an excitation of 390 nm and emission of 485 nm.

Ethylene Glycol Chitin Chitinase Assay

The degradation of ethylene glycol chitin was measured on agar plates. Luria-Bertani medium (Sambrook *et al.*, 1989. Molecular Cloning. A Laboroatory Manual. Cold

Spring Harbor Laboratory Press) agar plates were prepared containing 50 μg/ml ampicillin, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 0.05 % (w/v) ethylene glycol chitin and 0.01 % (w/v) trypan blue. 1 μl of a bacterial culture (grown for 16-20 h) was pipetted onto the surface of the agar and the plate incubated at 37 °C for 15.5 h. Degradation of the ethylene glycol chitin was shown by a clear halo around the bacteria, against a blue background.

Chitin Azure Chitinase Assay

10 mg of chitin azure was added to microcentrifuge tubes containing 950 μl of 200 mM sodium phosphate buffer pH 7.0. 50 μl of chitinase was added and the mixture incubated at 37 °C for 24 h on an end over end carousel mixer. The mixture was then centrifuged at 16000 x *g* for 10 min and the absorbance at 570 nm of the supernatant determined. Samples were compared to blanks containing sample buffer instead of chitinase.

Colloidal Chitin Chitinase Assay

Colloidal crab shell chitin was prepared essentially as described by Jeuniaux, C. (1966; Methods Enzymol., 8, 644-650.) using 2 g of practical grade crab shell chitin (Sigma Co.). Hydrolysis of colloidal chitin was determined in microtitre plates by measuring the decrease in optical density (O.D.) at 620 nm on an iEMS MF plate reader (Labsystems) at 37 °C. Briefly, 125 μl of 200 mM sodium phosphate buffer pH 7.0 was dispensed into microwells and 50 μl of a colloidal chitin suspension (40 mg/ml in 200 mM sodium phosphate buffer pH 7.0) added and pre-incubated at 37 °C for 15 min. The reaction was started by the addition of 25 μl of chitinase. The O.D. at 620 nm was measured every 5 min for 1 h. Prior to each measurement, the plate was shaken to ensure that the colloidal chitin was well dispersed. The

maximum rate of the reaction was calculated by the Labsystems Genesis software, after subtraction of a blank.

Lysozyme Activity

Lysozyme activity was determined by measuring the decrease in O.D. at 620 nm of a suspension of *Micrococcus lysodeikticus* cells. The assay was performed in a microtitre plate as described for colloidal chitin, except that the substrate was a suspension of lyophilised *Micrococcus lysodeikticus* (Sigma Co; 40 mg/ml) cells, in 200 mM sodium phosphate buffer pH 7.0. Hen egg white lysozyme (Sigma Co.) was used as a positive control for the assay.

Effect of pH on Activity and Stability

Chitinase activity was determined with 0.1 mM 4-Nitrophenyl-β-D-*N*,*N*'-Diacetylchitobiose over the range pH 4.4-9.0, using 100 mM sodium citrate buffers up to pH 5.35 and 200 mM sodium phosphate buffers to pH 9.0, as described above for the 4-nitrophenol assay.

The pH stability was determined over the range pH 3-10 by diluting purified chitinase 10 fold in either sodium citrate or sodium phosphate buffers, followed by incubation at 37 °C for 3 hours. After incubation, chitinase was then diluted 10 fold in 200 mM sodium phosphate buffer pH 7.0 and assayed for activity using 0.1 mM 4-Nitrophenyl-β-D-*N*,*N*'-Diacetylchitobiose at pH 7.0 and 37 °C, as described above.

Binding Studies

The binding of chitinase to crab shell chitin, chitin azure, colloidal chitin, chitosan, xylan, lichenan, heparin agarose, microgranular cellulose and *N,N'*-Diacetylchitobiose agarose was investigated by the addition of 1 ml of chitinase to 100 mg of the insoluble polysaccharides. The suspension was mixed continuously on a rotating end over end mixer at room temperature for 3 h and then centrifuged at 16000 x *g* for 10 min to remove the insoluble polysaccharides. The chitinase activity in the supernatant was determined and compared to a control to estimate the percentage bound.

Protein Assay

Protein concentrations were determined using a Pierce BCA protein assay kit. Assays were performed according to the manufacturer's instructions, with bovine serum albumin (BSA) being used to construct a standard curve. The absorbance at 570 nm of the samples and standards was measured on a Labsystems iEMS MF plate reader and the protein concentration calculated using the Labsystems Genesis software.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Chromatography fractions and purified chitinase samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described in Example 1, except that, after electrophoresis, gels were stained either with silver (see below) or with 0.1 % (w/v) Coomassie Brilliant Blue R-250 in 30 % (v/v) methanol and 10 % (v/v) acetic acid.

Isoelectric Focussing

Isoelectric focussing (IEF) was performed on purified chitinase samples to determine the pI point and purity of the protein. The method was performed using a Pharmacia Phast system with Pharmacia IEF 3-9 Phast gels. Samples in Milli-Q H_2O were loaded directly onto the gel using a 6 x 4 µl applicator. Pharmacia broad range pI standards were dissolved in 100 µl of Milli-Q H_2O and also loaded directly onto the gel. The program for IEF 3-9 on the Pharmacia Phast system was as follows. Pre-focussing at 2000 V, 2.5 mA, for 75 Vh ; apply sample applicator and run at 200 V, 2.5 mA, for 15 Vh ; remove sample applicator then run at 2000 V, 2.5 mA, for 410 Vh. Following electrophoresis, the IEF gel was fixed in 20% (w/v) trichloroacetic acid for 30 min and then silver stained, according to the method below.

Silver Staining of Polyacrylamide Gels

Silver staining was performed as described by Heukeshoven and Dernick (1985; *Electrophoresis* **6**: 103-112).

Western Blotting and Immunostaining.

Rabbit anti-*P. aeruginosa* 385 serum was prepared as described in Example 2.

NH_2-Terminal Amino Acid Analysis

Chitinase was subject to SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane for NH_2-terminal sequencing as described in Example 3.

Results

Subcellular Distribution of Chitinase in *Pseudomonas aeruginosa* 385

Cells of *P. aeruginosa* 385 were fractionated as described above. The various cell fractions obtained were analysed by SDS-PAGE (see Figure 9) and for chitinase activity (see Table 2). Inspection of the gel in Figure 9, shows that the different fractions have distinct protein compositions, suggesting that the fractionation has been successful. The growth medium, collected after harvesting of cells, clearly contains a number of proteins and *P. aeruginosa* is known to secrete a wide variety of enzymes. The protein concentration of the medium could not be calculated because one or more of the coloured compounds secreted by the cells interfered in the protein assay. No chitinase activity was detected in the medium, despite the expected requirement for an extracellular localisation of this enzyme. Furthermore, no chitinase activity was located in the periplasmic extract (cold shock extract) that was prepared. The majority of the chitinase was detected in the cytoplasmic fraction (66 %), with a small amount in the membrane fraction (2 %). We would not expect the cytoplasm to be the functionally significant location of this enzyme and it is possible that secretion of it is regulated in some manner, according to environmental conditions. The data we have obtained suggest that the chitinase is expressed constitutively: the bacteria have been grown in the absence of chitin or chitin degradation products that are known inducers of chitinase gene expression. It is possible that an inducer(s) could increase expression from this basal level.

Table 2 - Distribution of chitinase activity in subcellular fractions of *Pseudomonas aeruginosa* isolate 385

Sample	Chitinase activity (nmol/min/ml)	Total Units of chitinase activity	% Units of chitinase activity	Total protein (mg)	Specific activity of chitinase (U/mg)
TMK Extract	0	0	0	0.42	0
Cold shock extract	0	0	0	1.54	0
11000 x g supernatant	10.36	182	97.8	36.2	5.0
11000 x g pellet	0.84	4	2.2	2.70	1.5
Cytoplasmic fraction	7.6	123	66.1	21.8	5.6
Membrane fraction	2.32	3	1.6	8.0	0.38

The fractions are as follows : TMK extract - obtained from TMK buffer wash prior to cold shock treatment; Cold shock extract - resulting from cold shock and corresponding to periplasmic proteins; 11000 x *g* supernatant - supernatant resulting from 11000 x *g* centrifugation of sonicated cells, following cold shock treatment; 11000 x *g* pellet - pellet resulting from 11000 x *g* centrifugation of sonicated cells, following cold shock treatment; Cytoplasmic fraction - supernatant after 200,000 x *g* centrifugation of 11000 x *g* supernatant, described above; Membrane fraction - pellet after 200,000 x *g* centrifugation of 11000 x *g* supernatant, described above. For calculation of % Units of chitinase activity, the sum of the Units in the 11000 x *g* supernatant and 11000 x *g* pellet samples (186 Units) was defined as 100 %.

Purification of Chitinase from *Pseudomonas aeruginosa* 385

Chitinase was purified from the soluble protein extract (periplasmic and cytoplasmic) of *Pseudomonas aeruginosa* 385 using a chitin binding method. SDS-PAGE analysis (see Figure 10) shows that most of the proteins in the extract did not bind to chitin and were therefore washed from the substratum with the use of 200 mM sodium phosphate buffer pH 7.0. After three washes, no unbound protein remained and the chitinase was eluted from the column in 70 % (v/v) ethylene glycol. This gave a pure protein of 58 kDa by SDS-PAGE analysis (see Figure 10). A sample of the chitin, after the 70 % (v/v) ethylene glycol wash, was also analysed by SDS-PAGE and this showed that some chitinase was still bound, along with another protein of 42 kDa. A protein assay and chitinase assay was performed on the purified chitinase. 1.61 mg of chitinase, with a specific activity of 106.3 nmoles/min/mg of protein, was recovered from 5 ml of soluble protein extract using 500 mg of purified crab shell chitin. The overall recovery was 35 % of the chitinase activity measured in the soluble protein extract, with a purification factor of 29.5.

A Western blot of the purified chitinase was performed and immunostained with rabbit anti-*P. aeruginosa* 385 serum (see Figure 11). The chitinase was detected by the antiserum and showed a strong antigenic response at 58 kDa. There is also a slight antigenic response at 10 kDa, which is not detected by SDS-PAGE and silver staining (see Figure 10), indicating that it is a minor contaminant.

The NH_2-terminal amino acid sequence of the purified chitinase was determined as (S,G or A)-R-E-D-A. This is identical to the sequence of amino acid residues 12-16 (inclusive) of the chitinase of *P. aeruginosa* strain PA01 (obtained from www.pseudomonas.com), suggesting that some proteolysis has occurred to remove the eleven N-terminal residues.

Determination of the Chitinase pI Point

Figure 12 shows a silver stained Pharmacia IEF Phast gel 3-9, of the chitinase. The pI was estimated as 5.2 from the gel which is in good agreement with the pI of 5.11 calculated from the chitinase sequence of strain PAO1 *P. aeruginosa* (www.pseudomonas.com), using protein sequence analysis software (DNASIS version 2.5). The IEF gel showed a pure chitinase band with no other proteins visible on the gel.

pH Activity Profile and Stability

The activity of purified chitinase as a function of pH was studied using 4-Nitrophenyl-β-D-*N,N'*-Diacetylchitobiose as a substrate and is shown in Figure 13. The graph shows that maximum activity was attained at pH 6.75, with over 90 % activity retained in the range pH 6.5-7.1 and 50 % in the range pH 6.0-7.5. Below pH 6.0 and above pH 8.0, activity fell rapidly with less than 10 % activity detected below pH 5.0 and above pH 8.6. The results show a neutral pH optimum, unlike other chitinases derived from fungi and plants which show acidic pH optimum.

The pH stability of chitinase was determined in the range pH 3-10 after incubation at 37°C for 3 h. Residual activity was determined using 4-Nitrophenyl-β-D-*N,N'*-Diacetylchitobiose. Table 3 shows that the enzyme was inactive at pH 3.0. At pH 4.0 the activity was reduced by 46 %. Throughout the range pH 5-10 the enzyme was stable under the conditions employed.

Table 3: pH stability of *Pseudomonas aeruginosa* isolate 385 native and recombinant chitinases, measured using 4-Nitrophenyl-β-D-*N,N'*-Diacetylchitobiose.

pH	% Chitinase activity relative to control	
	Native chitinase	Recombinant chitinase
3	0	0
4	54	42
5	100	93
6	100	93
7	100	91
8	100	96
9	100	96
10	97	78

The chitinase was incubated at 37 °C for 3 h, in various pH buffers and then the activity measured after dilution in assay buffer (pH 7.0). The control is chitinase diluted in assay buffer and measured immediately.

Substrate Specificity

The chitinase showed activity against ethylene glycol chitin, chitin azure, colloidal chitin and a range of synthetic substrates. Table 4 shows the activity against a range of chitin substrates; the chitinase was active against all substrates tested. Table 5 shows the activity against a range of methlyumbelliferyl β-1, 4 linked glucosamine oligosaccharides. There was no activity against 4-Methylumbelliferyl *N*-Acetyl-β-D-Glucosaminide, indicating a lack of exochitinase (β-1,4 -hexosaminidase) activity. Activity was measured against 4-Methylumbelliferyl-β-D-*N,N'*-Diacetylchitobioside, 4-Methylumbelliferyl-β-D-*N,N',N''*-Triacetylchitotrioside , and 4-Methylumbelliferyl-β-D-*N,N',N'',N'''*-Tetraacetylchitotetraoside. These all represent oligosaccharide analogues of two or more sugars and demonstrates the endo-splitting nature of the chitinase activity. The highest activity was against 4-

Methylumbelliferyl-β-D-*N*,*N'*-Diacetylchitobioside, which suggests that the primary function of the enzyme is the removal of diacetylchitobiose from the non-reducing end of chitin or chitodextrins.

Table 4: The activity of *Pseudomonas aeruginosa* isolate 385 native and recombinant chitinases against a range of chitin substrates.

Substrate	Chitinase activity	
	Native chitinase	Recombinant chitinase
Chitin azure[a]	1.12 U/mg	4.87 U/mg
Colloidal chitin[b]	870 U/mg	1190 U/mg
Ethylene glycol chitin[c]	+	+

a. 1 Unit = ΔO.D. 570 nm of 1.0 per 24 h

b. 1 Unit = ΔO.D. 620 nm of 1.0 per min

c. + = zone of clearing observed around colonies of bacteria grown on agar plates containing ethylene glycol chitin.

Table 5: Substrate specificity of *Pseudomonas aeruginosa* isolate 385 native and recombinant chitinases against a range of fluorogenic *N*-acetyl glucosamine derivatives.

Substrate	Mean (n=3) Fluorescent Units/mg chitinase	
	Native chitinase	Recombinant chitinase
4-Methylumbelliferyl *N*-Acetyl-β-D-Glucosaminide	0	0
4-Methylumbelliferyl-β-D-*N*,*N'*-Diacetylchitobioside	7.24×10^5	10.4×10^5
4-Methylumbelliferyl-β-D-*N*,*N'*,*N''*-Triacetylchitotrioside	4.93×10^5	7.09×10^5
4-Methylumbelliferyl-β-D-*N*,*N'*,*N''*,*N'''*-Tetraacetylchitotetraoside	1.86×10^5	3.74×10^5

Many chitinases display lysozyme activity and are able to depolymerise the peptidoglycan of bacterial cell walls by the hydrolysis of β-1,4 linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine. However, the chitinase from *Pseudomonas aeruginosa* 385 did not display any lysozyme activity, as determined by the lack of lysis of *Micrococcus lysodeikticus* cells.

Kinetic Studies

The K_m, k_{cat} and specificity constant (k_{cat}/K_m,)were calculated for the substrates 4-Nitrophenyl-β-D-*N*,*N*'-Diacetylchitobiose and 4-Nitrophenyl-β-D-*N*,*N*',*N*''-Triacetylchitotriose and are shown in Table 6. The specificity constants for both substrates are similar, but the K_m's and k_{cat}'s differ by an order of magnitude. The lowest K_m is that for 4-Nitrophenyl-β-D-*N*,*N*',*N*''-Triacetylchitotriose and the highest turnover number is for 4-Nitrophenyl-β-D-*N*,*N*'-Diacetylchitobiose. Although the specificity constants are comparable, indicating similar substrate preference, the kinetic data suggest that the enzyme may have evolved to maximise rates against the removal of diacetylchitobiose from the non-reducing end of chitin or chitodextrins by increasing both K_m and k_{cat}.

Table 6: Kinetic parameters for *Pseudomonas aeruginosa* isolate 385 native and recombinant chitinases using the substrates 4-Nitrophenyl-β-D-*N,N'*-Diacetylchitobiose and 4-Nitrophenyl-β-D-*N,N',N''*- Triacetylchitotriose.

Substrate	K_m (mM)		K_{cat} (S^{-1})		*Kcat/Km* ($S^{-1}M^{-1}$)	
	Native chitinase	Recomb chitinase	Native chitinase	Recomb chitinase	Native chitinase	Recomb chitinase
4-Nitrophenyl-β-D-*N-N'*-Diacetylchitobiose	4.28	4.38	1.7	4.3	404	970
4-Nitrophenyl-β-D-*N,N',N''*-Triacetylchitotriose	0.48	0.48	0.16	0.41	341	868

Binding Studies

Table 7 shows the results of investigations into the binding of chitinase to a number of different insoluble carbohydrate substrates. 100% of the chitinase was bound to three of the chitin substrates, however only 25% bound to chitosan. Over 80% of the chitinase bound to lichenan and microgranular cellulose but less than 45% bound to xylan, heparin agarose and *N,N'*-Diacetylchitobiose agarose.

Table 7: Binding of native and recombinant chitinases to insoluble polysaccharides. Chitinase activity was measured using 4-Nitrophenyl-β-D-*N,N'*-Diacetylchitobiose.

Substrate	% Chitinase activity bound	
	Native chitinase	Recombinant chitinase
Purified crab shell chitin	100	99
Chitin azure	100	93
Colloidal chitin	100	92
Chitosan	25	17
Xylan	42	36
Lichenan	88	56
Heparin cellulose	35	11
Microgranular cellulose	81	58
N,N'-Diacetylchitobiose agarose	36	22

Example 5 - Cloning and Expression of a Chitinase from *Pseudomonas aeruginosa* isolate 385

Bacterial Strains

Pseudomonas aeruginosa strains and clinical isolates used in this example are described in Table 8. The clinical isolates of *Pseudomonas aeruginosa* (except J1532), were obtained by plating sputum from cystic fibrosis patients on nutrient agar. The serotype of the clinical isolates was determined according to the International Antigenic Typing Scheme (IATS) by the Public Health Laboratory Service, Colindale, London, U.K.

Table 8 - *Pseudomonas aeruginosa* strains and isolates used in this example

Strain / isolate number	Equivalent ATCC strain number	Serotype	Source
NCTC11440	33348	1	NCTC (PHLS)
NCTC11441	33349	2	NCTC (PHLS)
NCTC11442	33350	3	NCTC (PHLS)
NCTC11443	33351	4	NCTC (PHLS)
NCTC11444	33352	5	NCTC (PHLS)
NCTC11446	33353	6	NCTC (PHLS)
NCTC11445	33354	7	NCTC (PHLS)
NCTC11447	33355	8	NCTC (PHLS)
NCTC11448	33356	9	NCTC (PHLS)
NCTC11449	33357	10	NCTC (PHLS)
NCTC11450	33358	11	NCTC (PHLS)
NCTC11451	33359	12	NCTC (PHLS)
NCTC11452	33360	13	NCTC (PHLS)
NCTC11453	33361	14	NCTC (PHLS)
NCTC11663	33362	15	NCTC (PHLS)
NCTC11455	33363	16	NCTC (PHLS)
NCTC11456	33364	17	NCTC (PHLS)
385	-	2	AIMI clinical isolate

Strain / isolate number	Equivalent ATCC strain number	Serotype	Source
ATCC27853	27853	6	NCTC (PHLS)
373	-	1	AIMI clinical isolate
398	-	PA	AIMI clinical isolate
422	-	NT	AIMI clinical isolate
423	-	6	AIMI clinical isolate
459	-	6	AIMI clinical isolate
522	-	3	AIMI clinical isolate
526	-	1	AIMI clinical isolate
537	-	PA	AIMI clinical isolate
J1532	-	N.d.	J. McGovan, Univ. of Edinburgh, U.K.

Abbreviations used : NCTC(PHLS) - National Collection of Type Cultures (Public Health Laboratory Service), Colindale, London, U.K.; ATCC - American Type Culture Collection, Rockville, Maryland, U.S.A.; AIMI - Australian Institute for Mucosal Immunology, Royal Newcastle Hospital, Newcastle, New South Wales, Australia; PA - Polyagglutinating. Reactivity with all serotypes; NT - Non-typeable. No reaction with any serotype; N.d. - Not determined.

Growth of Bacteria

Pseudomonas aeruginosa stocks were stored at -80 °C in tryptone soya broth (TSB) containing 20 % (v/v) glycerol. *Escherichia coli* stocks were stored at -80 °C in either M9 minimal medium or Luria-Bertani (LB) broth (Sambrook *et al.*, 1989. Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press), supplemented with a final concentration of 50 μg/ml ampicillin and containing 40 % (v/v) glycerol.

Pseudomonas aeruginosa strains and clinical isolates were grown in 50 ml tubes with 20 ml TSB at 37 °C in an orbital shaker (250 rpm) for 16-20 h.

Recombinant *Escherichia coli* strains were grown in either M9 minimal medium or LB broth, supplemented with a final concentration of 50 μg/ml ampicillin.

For culture on plates, media were solidified by the incorporation of 1.5 % (w/v) agar.

Standard Molecular Biology Techniques

Standard molecular biology techniques were carried out according to the texts, Molecular Cloning. A Laboratory Manual (Sambrook *et al.* 1989. Cold Spring Harbor Laboratory Press) and Current Protocols in Molecular Biology (Ausubel *et al.* 1999. John Wiley & Sons).

Extraction of Genomic DNA

Genomic DNA was extracted from *Pseudomonas aeruginosa* cells using Qiagen genomic 100-G tips with a Qiagen genomic DNA buffer set, according to the manufacturer's instructions. The quality of the DNA was assessed by measuring the ultra-violet (UV) absorbance spectrum between 230 nm and 330 nm. The absorbance at 260 nm was used to quantify the DNA.

Southern Blotting and Hybridisation

Probes were synthesised using a Boehringer Mannheim PCR (polymerase chain reaction) DIG (digoxigenin) probe synthesis kit, according to the manufacturer's

instructions, with 10 ng *Pseudomonas aeruginosa* 385 genomic DNA as template. For restriction endonuclease fragment analysis of *P. aeruginosa* 385 genomic DNA, probes corresponding to 5' and 3' fragments of the chitinase gene (see below) were synthesised. For detection of the chitinase gene in different *P. aeruginosa* isolates, a probe consisting of the full coding sequence of the isolate 385 gene was produced, using the recombinant pKK223-3 plasmid carrying this gene (see below) as a template. The PCR reactions were analysed on 1 % (w/v) agarose gels and the relevant products excised and purified using a Qiagen Qiaquick gel extraction kit. The DNA was stored at -20 °C and an aliquot heat denatured before addition to pre-hybridised membranes.

1 µg of genomic DNA was digested with 2 units of restriction endonuclease (either *Cla*I, *Xho*I, *Bss*HII, *Sal*I, *Bgl*II (all Promega) or *Sma*I (MBI Fermentas)), according to the manufacturer's recommendations, for 16-17 h. The restriction fragments were separated on a 0.7 % (w/v) agarose gel, cast and run in 0.5 x TBE buffer (Sambrook *et al.*, 1989. Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press). The DNA fragments were transferred to a positively charged nylon membrane (Boehringer Mannheim) using a Bio-Rad TransBlot at 3.55 mA / cm^2 gel for 10-15 min, denatured by incubation of the membrane on filter paper soaked in 0.4 M NaOH and cross-linked by UV irradiation in a Stratalinker 2400 (Stratagene; auto cross-link mode). Membranes were pre-hybridised for 1-2 h at 37 °C in DIG Easy Hyb buffer (Boehringer Mannheim) and then 2.5 µl DIG-labelled probe added in the same buffer. Hybridisation was at 37 °C for approximately 16 h. The blots were washed twice with 2 x SSC, 0.1 % (w/v) sodium dodcecyl sulphate (SDS) (Sambrook *et al.*, 1989. Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press) at 25 °C, then twice with 0.5 x SSC, 0.1 % (w/v) SDS at 68 °C. Hybridising fragments were detected using an anti-DIG-alkaline

phosphatase conjugate antibody with a Boehringer Mannheim nucleic acid detection kit, according to the manufacturer's instructions.

Extraction of Plasmid DNA

Plasmid DNA was extracted from recombinant *E. coli* strains using either a Qiagen Qiaprep spin plasmid miniprep kit or a Qiagen Qiafilter plasmid midi kit, according to the manufacturer's recommendations.

Oligonucleotide Synthesis

Oligonucleotide primers for PCR and DNA sequencing were synthesised by Sigma-Genosys, U.K.

Nucleotide Sequencing and Sequence Analysis

Nucleotide sequencing was carried out at the Microchemical Facility, Babraham Institute, Cambridge, U.K. Full sequence data was obtained for both strands of DNA, using a primer-walking strategy to obtain overlapping sequence fragments. These fragments were compiled using DNASIS v2.5 software. This software was also used for all other sequence analyses.

Polymerase Chain Reaction

PCR was performed using either Pfu DNA polymerase (Stratagene) or Taq DNA polymerase (Promega or Advanced Biotechnologies), with the buffer supplied by the manufacturers. The 10 x reaction buffer for Taq DNA polymerase from Promega incorporated 1.5 mM $MgCl_2$. Reactions were performed in a total volume of 25 µl

and incorporated 0.2 mM each deoxyribonucleoside triphosphate (dNTP), 0.4 μM each primer and 1 unit DNA polymerase. Reactions were overlaid with 25 μl mineral oil and performed using a Hybaid Touchdown thermal cycler.

Taq DNA polymerase from Advanced Biotechnologies was obtained as Reddy-Load PCR mix (1.5 mM $MgCl_2$) which includes buffer and dNTPs. Therefore, the only additions made to this were primers (each at 0.4 μM) and template DNA.

Cloning of the Chitinase Gene

The information obtained from the *Pseudomonas aeruginosa* PA01 genome sequence (www.pseudomonas.com) allowed us to design primers for amplification by PCR of the 5' and 3' ends of the chitinase gene from *P. aeruginosa* isolate 385. These primers were designed upon the sequence of the *P. aeruginosa* PA01 genome, such that they amplified the ends of the chitinase gene, along with flanking 5' or 3' DNA. Sequencing of the products obtained allowed us to design primers to amplify only the coding region of the gene from *P. aeruginosa* 385. These primers had the following sequences ; 5'-ATGATCAGGATCGACTTTTCCCAG-3' and 5'-TCAGCGCAGCGGCCGCCAGA-3'. Amplification using these two primers was achieved using the following thermal cycling program: 30 cycles of, denaturing at 94 °C for 45 s, annealing at 63 °C for 1 min and extension at 72 °C for 2.5 min. It was not possible to obtain a PCR product corresponding to the coding region when genomic DNA isolated from *P. aeruginosa* 385 was used as a template. Therefore, we used DIG-labelled probes of the 5' and 3' products described above to carry out Southern blotting experiments on genomic DNA restricted with several different endonucleases (see methods). The chitinase gene was located on a *Bss*HII fragment of approximately 2500 base pairs (bp). Therefore, genomic DNA was digested with *Bss*HII and the fragments in the 2500 bp size range were purified after separation on

a 0.7 % (w/v) agarose gel. These were then used as a template in PCR reactions to yield the chitinase gene. A PCR product corresponding to the full coding region of the chitinase gene was generated using Taq DNA polymerase. The product was purified from an agarose gel using a Qiagen Qiaquick gel extraction kit and the ends polished using T4 DNA polymerase. The product was phosphorylated using T4 polynucleotide kinase and then ligated with pKK223-3 expression vector that had been digested with *Sma*I and dephosphorylated with calf intestinal alkaline phosphatase. The ligation mixture was transformed into *E. coli* JM109 cells and positive clones were identified by PCR analysis of extracted plasmids. Two independent clones were sequenced fully to confirm that no errors had been introduced during PCR.

Production of Recombinant Chitinase

A loopful of glycerol stock of *E. coli* JM109 cells, carrying the pKK223-3 plasmid with the cloned chitinase gene, was streaked onto an M9 minimal medium agar plate, including ampicillin. The plate was incubated at 37 °C for 24 h and a single colony was then used to inoculate 5 ml of M9 medium, containing ampicillin. This starter culture was grown at 37 °C for 16 h, with shaking at 200 rpm. This was then used to inoculate two or three 2-litre flasks, each containing 600 ml of LB medium supplemented with ampicillin. These flasks were incubated as above. When the optical density (O.D.) of the culture, measured at 600 nm, was approximately 0.7 (t=0), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and growth continued for 4 h. Samples were taken at t=0 and t=4h for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Subcellular Fractionation of Recombinant *E. coli* Expressing Chitinase

Cells were harvested from cultures by centrifugation at 4000 x *g* for 1 h 45 min and washed with one volume of 25 mM sodium phosphate buffer pH 7.0. Periplasmic proteins were extracted by a cold osmotic shock treatment. The cells from 1.8 litres of culture were resuspended with 45 ml 10 mM Tris-HCl, 30 mM NaCl pH 7.3 and then collected by centrifugation at 11000 x *g* for 5 min. The cell pellet was resuspended in 60 ml of the same buffer and centrifugation repeated as above. Washed cells were suspended in 30 ml 33 mM Tris-HCl pH 7.3 and then 30 ml 33 mM Tris-HCl, 40 % (w/v) sucrose, 2 mM EDTA pH 7.3 added. The tube was agitated gently for 5 min and the cells then harvested by centrifugation at 11000 x *g* for 10 min and resuspended with 60 ml ice-cold Milli-Q water. The cells were incubated on ice for 3 min then 1.2 ml 50 mM $MgCl_2$ added, prior to a further 10 min incubation on ice. The osmotically shocked cells were collected by centrifugation at 11000 x g for 10 min. The supernatant, containing periplasmic proteins, was removed and retained and the cell pellet resuspended with 60 ml 25 mM sodium phosphate buffer pH 7.0, containing 1 mM 1-(2-aminoethyl)benzenesulfonylfluoride-HCl (AEBSF protease inhibitor). The cells were then sonicated on ice as two 30 ml batches, using a Sanyo Soniprep 150 (19 mm probe) at an amplitude of 6 μm with 30 s sonication followed by 1 min standing, for 25 cycles. Cell debris and intact cells were removed by centrifugation at 11000 x *g* for 45 min. The supernatant was retained and the pellet resuspended with 20 ml 25mM sodium phosphate buffer pH 7.0. The 11000 x *g* supernatant (cytoplasmic proteins and membranes) was centrifuged further at 200,000 x *g* for 90 min and the supernatant (cytoplasmic proteins) collected. The pellet (membranes) was resuspended in 9 ml 25 mM sodium phosphate buffer pH 7.0. Each of the fractions obtained above was analysed by SDS-PAGE and assayed for chitinase activity.

For routine purification of recombinant chitinase, the osmotic shock procedure was omitted. In this case, the harvested and washed cells from 1.2 litres of culture were resuspended with 40 ml 25 mM sodium phosphate buffer pH 7.0, containing 1 mM AEBSF and sonicated in two 20 ml batches as above. The sonicated material was centrifuged at 11000 x *g* for 30 min and the supernatant removed. This was further centrifuged at 200,000 x *g* for 90 min and the resulting supernatant soluble protein extract (cytoplasmic and periplasmic) was used for purification of recombinant chitinase.

Purification of Recombinant Chitinase

Purification by Chitin Binding

Five 100 mg aliquots of purified crab shell chitin (Sigma Co.), were mixed thoroughly in a microcentrifuge tube with 1 ml of soluble protein extract from *E. coli* JM109 cells expressing recombinant chitinase. The mixture was incubated for 17 h at room temperature on an end over end carousel mixer. The tube was then centrifuged at 16000 x *g* for 5 min and the supernatant removed. The chitin was washed three times with 1 ml of 200 mM sodium phosphate buffer pH 7.0 for 30 min, with centrifugation as above after each wash. Chitinase was then eluted from the chitin with 2 x 500 µl of 80 % (v/v) ethylene glycol (in 200 mM sodium phosphate buffer pH 7.0), for 30 min at room temperature. The chitin was collected after each elution by centrifugation as above and the supernatants retained. The first 500 µl 80 % (v/v) ethylene glycol washes of each aliquot was pooled to give 2.5 ml of sample. This was buffer-exchanged into 50 mM sodium phosphate buffer pH 7.0 using a Pharmacia PD10 column (see below) to give a final volume of 3.5 ml.

The purity of the chitinase was analysed by SDS-PAGE, isoelectric focussing (IEF) and Western blotting as described in Example 4.

Purification by Chromatography

Buffer-exchange of Samples

This was carried out as described in Example 4.

Hydrophobic Interaction Chromatography (HIC)

3 x 2.5 ml of soluble protein extract from *E. coli* JM109 cells expressing recombinant chitinase were buffer-exchanged into 50 mM sodium phosphate buffer pH 6.0, containing 3 M NaCl, by use of a PD10 column. After buffer-exchange, samples were clarified by centrifugation at 11000 x *g* for 20 min to remove insoluble material. 10 ml of the pooled PD10 eluants was loaded at 3 ml/min onto 25 ml of Phenyl Sepharose High Performance media (packed in a Pharmacia XK 16/20 column), equilibrated with 125 ml of 50 mM sodium phosphate buffer pH 6.0 containing 3 M NaCl, using a BioCad Vision Chromatography Workstation (PE Biosystems). Unbound material was washed through the column at 3 ml/min with 125 ml of 50 mM sodium phosphate buffer pH 6.0 containing 3 M NaCl. Elution was obtained at a flow rate of 3 ml/min by a 50 mM sodium phosphate buffer pH 6.0 containing 3 M NaCl to 50 mM sodium phosphate buffer pH 6.0 gradient over 5 column volumes, followed by a 125 ml Milli-Q water wash. The eluate was continuously monitored at 280 nm and 9 ml fractions were collected throughout the gradient and Milli-Q water wash using a chilled (4 °C) fraction collector. A chitinase assay and SDS-PAGE analysis was performed on each fraction. Fractions were pooled according to the greatest activity and protein purity.

Anion-exchange Chromatography

9 ml aliquots of partially purified, pooled chitinase samples, obtained from HIC, was loaded onto a 1 ml Pharmacia Mono Q column, using a BioCad Vision at 1 ml/min. Unbound material was washed through the column with 10 ml of 50 mM sodium phosphate buffer pH 7.0. Protein was eluted by using a 0-1 M NaCl gradient in 50 mM sodium phosphate buffer pH 7.0, over 20 column volumes at 1 ml/min. The eluate was continuously monitored at 280 nm and 1 ml fractions were collected throughout, using a chilled (4 °C) fraction collector. Each fraction was tested for protein content, chitinase activity and analysed by SDS-PAGE. Fractions containing chitinase were pooled according to the greatest activity and protein purity.

Gel Filtration Chromatography

Pooled chitinase fractions, obtained by anion-exchange chromatography, were purified by gel filtration chromatography using a BioCad Vision. Samples were loaded in 500 µl aliquots onto a Pharmacia Superdex 200 column (25 ml), pre-equilibrated with 125 ml of 25 mM sodium phosphate buffer pH 7.0. Samples were eluted in 25 ml of the same buffer at 3 ml/min. The eluate was constantly monitored at 280 nm and 210 nm. 1 ml fractions were collected throughout using a chilled (4 °C) fraction collector. Each fraction was analysed by SDS-PAGE and for chitinase activity and protein content.

Chitinase Assays

The chitinolytic activity of samples was determined as in Example 4.

In the ethylene glycol chitin chitinase assay, 1 µl of a bacterial culture of *E. coli* JM109 cells carrying the pKK223-3 plasmid with the cloned chitinase gene (grown for 16-18 h) was pipetted onto the surface of the agar and the plate incubated at 37 °C for 15.5 h. Degradation of the ethylene glycol chitin was shown by a clear halo around the bacteria, against a blue background. As a negative control, a culture of *E. coli* JM109 cells carrying an unmodified pKK223-3 plasmid was included. No halo was observed around the resulting bacterial colony for this control.

Lysozyme Activity, Effect of pH on Activity and Stability, Binding Studies, Protein Assay, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis, Isoelectric Focussing, Silver Staining of Polyacrylamide Gels, Western Blotting and Immunostaining, and NH_2-Terminal Amino Acid Analysis.

All of these were determined as in Example 4.

Results

Cloning and Expression of the Chitinase Gene

Figure 14 shows the sequence obtained for the chitinase gene of *P. aeruginosa* isolate 385, along with the flanking sequence obtained by sequencing of the 5' and 3' PCR products described above. We have designated this gene *chi*A. The sequence of Figure 14 is 99 % identical (1844 out of 1858 bases) to that of the corresponding fragment of the *P. aeruginosa* PA01 genome (obtained from www.pseudomonas.com). As seen for the *P. aeruginosa* PA01 chitinase gene, the open reading frame begins with an ATG and encodes a polypeptide of 483 amino acid residues with a predicted Mr of 53 kDa. The G+C content, codon usage and occurrence of G/C bases at each codon position are all typical of *P. aeruginosa*.

The *chiA* nucleotide sequence is 99.6 % identical to that of the PA01 gene and the deduced amino acid sequence is 100 % identical. The protein sequence was compared with entries in public sequence databases and the greatest similarity was found to be with ChiC, a chitinase of *Serratia marcescens* (53 % identity). The level of identity of the corresponding genes is 63 %. Analysis of the protein sequence using the SignalP signal peptide prediction program (Nielsen *et al.* 1997. *Protein Eng.* **10**: 1-6) predicted that the protein lacks a typical N-terminal signal peptide for secretion. This was also found for the *S. marcescens* ChiC but despite this, ChiC is secreted into the extracellular medium. We have obtained no indication that the *P. aeruginosa* enzyme is secreted into the medium under the growth conditions used. Indeed, most of the protein was found to be located in the cytoplasm. The proposed function of this protein would indicate an extracellular location and so it is likely that *P. aeruginosa* possesses a secretion system similar to that responsible for ChiC secretion in *S. marcescens*. This secretion may be regulated according to growth conditions.

In order to determine whether the *chiA* gene is widespread in *P. aeruginosa* isolates, we performed Southern blot analysis using *Bss*HII restricted genomic DNA from 17 serotype strains, one type strain (ATCC27853) and 10 clinical isolates. The DIG-labelled probe employed, corresponded to the full coding region of the gene. The results are shown in Table 9.

Table 9 - Results of Southern blotting experiments to determine whether the *chi*A gene is present in various *Pseudomonas aeruginosa* strains and isolates. Abbreviations are as described for Table 8.

Strain / isolate	Serotype	Size of hybridising band on Southern blot (base pairs)
NCTC11440	1	None
NCTC11441	2	2350
NCTC11442	3	2350
NCTC11443	4	2350
NCTC11444	5	2350
NCTC11446	6	2350
NCTC11445	7	2350
NCTC11447	8	2350
NCTC11448	9	2350
NCTC11449	10	2350
NCTC11450	11	2310
NCTC11451	12	3720
NCTC11452	13	4690
NCTC11453	14	2630
NCTC11663	15	2630
NCTC11455	16	2590
NCTC11456	17	2590
385	2	2350
ATCC27853	6	2225
373	1	2555
398	?	2555
422	?	2225
423	6	2330
459	6	2330
522	3	2430
526	1	2400
537	?	2950
J1532	?	2530

The *chi*A gene was identified in all clinical isolates and in all other strains, except strain NCTC11440 (serotype 1). This result was confirmed by the inability to detect

chitinase activity in cell extracts of this strain and the failure to amplify 5' and 3' fragments of the *chi*A gene by PCR, when using genomic DNA from this strain as template. We confirmed the identity of NCTC11440 as *P. aeruginosa* by chemical tests and by the successful PCR amplification from genomic DNA of genes encoding the highly conserved *P. aeruginosa* outer membrane proteins, OprF, OprL and OprI. The lack of a *chi*A gene is not common to all serotype 1 strains since some of the clinical isolates studied are of this serotype and still possess this gene. We currently have no explanation for the lack of *chi*A in strain NCTC11440 and whether this strain differs in any other respects. It would appear however, that the gene is widespread amongst *P. aeruginosa* isolates.

The cloned chitinase gene was expressed in *E. coli* from the pKK223-3 expression plasmid. Following induction of expression, a protein of apparent molecular weight 58 kDa accumulated to become a significant component of the total cell protein, as judged by SDS-PAGE analysis of whole cell lysates (Figure 15). This protein has the same apparent molecular weight, measured by SDS-PAGE, as the native chitinase protein. Cells expressing the protein were fractionated as described and the various cell fractions were assayed for chitinase activity and analysed by SDS-PAGE (see Table 10 and Figure 15). The determined distribution of the protein is very similar to that determined for the chitinase in *P. aeruginosa* 385 cells, with the majority being localised in the cytoplasm. The soluble protein extract, containing cytoplasmic and periplasmic proteins was, therefore, chosen as the source for purification of recombinant chitinase. This circumvents the need for routine extraction of periplasmic proteins from cells.

Table 10 - Distribution of chitinase activity in subcellular fractions of *E. coli* JM109 cells, expressing recombinant chitinase.

Sample	Chitinase activity (nmol/min/ml)	Total Units of chitinase activity	% Units of chitinase activity	Total protein (mg)	Specific activity of chitinase (U/mg)
Periplasmic extract	19.92	1185	4.8	211.2	5.6
Crude sonicate	382.44	23711	95.2	979.6	24.2
11000 x g supernatant	400.92	23494	94.4	826.3	28.4
11000 x g pellet	20.72	487	1.9	133.2	3.7
Cytoplasmic fraction	346.44	19262	77.4	545.4	35.3
Membrane fraction	277.28	2995	12.0	202.0	14.8

The fractions are as follows : Periplasmic extract – obtained by cold osmotic shock treatment ; Crude sonicate – sonicated cells, following cold osmotic shock; 11000 x *g* supernatant – supernatant following 11000 x *g* centrifugation of crude sonicate sample; 11000 x *g* pellet – pellet following 11000 x *g* centrifugation of crude sonicate sample; Cytoplasmic fraction – supernatant after 200,000 x *g* centrifugation of 11000 x *g* supernatant, described above; Membrane fraction – pellet after 200,000 x *g* centrifugation of 11000 x *g* supernatant, described above. For calculation of % Units of chitinase activity, the sum of the Units in the periplasmic extract and the crude sonicate sample (24896 Units) was defined as 100 %.

Purification of Recombinant Chitinase by Chitin Binding

Recombinant chitinase was purified from the soluble protein extract of *E. coli* JM109 cells, expressing recombinant chitinase, using a chitin binding method. SDS-PAGE

results (Figure 16) show that most of the proteins did not bind to the chitin and were therefore eluted in the phosphate buffer washes. To ensure that the majority of the chitinase was eluted from the chitin, two 80 % (v/v) ethylene glycol washes were performed, both of which yielded a protein with a molecular weight of 58 kDa. SDS-PAGE analysis of chitin, following the ethylene glycol elutions, revealed that some chitinase was still bound to the substratum. The first 80 % (v/v) ethylene glycol eluates were used for further characterisation. A Western blot was performed on the purified chitinase (Figure 11). Chitinase was detected by rabbit anti-*P. aeruginosa* 385 serum as shown by an antigenic response at 58 kDa. No other bands were detected showing that the preparation was antigenically pure.

The NH_2-terminal sequence of the purified chitinase was determined as M-I-R-I-D. This represents amino acid residues 1-5 (inclusive) of the chitinase, as shown in Figure 14.

Figure 12 shows a Pharmacia IEF Phast gel 3-9, of recombinant chitinase. The protein focussed along side the 5.2 pI marker. The estimated pI (DNASIS version 2.5 software) for chitinase was 5.11, which gave good correlation with the results obtained here. The IEF gel shows a very pure chitinase preparation with no other proteins visible in the sample.

Protein analysis of the purified sample gave a concentration of 1.17 mg/ml. The chitinase activity of the sample was 192 nmoles/min/ml, which gave a specific activity of 164.8 nmoles/min/mg of protein. The yield of chitinase was 4.1 mg from 5 ml of soluble protein extract, which represents a recovery of 36.4 % of the chitinase activity measured in the soluble protein extract and a purification factor of 4.9.

Purification of Recombinant Chitinase by Chromatography

Hydrophobic Interaction Chromatography

Soluble protein extract from *E. coli* JM109 cells expressing recombinant chitinase was buffer-exchanged into 50 mM sodium phosphate pH 7.0 containing 3M NaCl. This caused a salting out of protein, which was removed by centrifugation. Chitinase and protein assays were performed on the soluble protein extract, PD10 buffer-exchange supernatant and PD10 buffer-exchange precipitate (Table 11). Salting out caused a loss of 1 % of the chitinase activity. Recombinant chitinase was purified by hydrophobic interaction chromatography (HIC). The elution profile is shown in Figure 17. Fractions were analysed by SDS-PAGE (Figure 18). The results show that majority of the protein eluting in the water wash (fractions 49 and 50) had a molecular weight of 58 kDa. Chitinase assays showed that most of the activity was also present in these fractions, and these were pooled. A protein assay was performed on the pooled sample, and gave a protein concentration of 0.679 mg/ml. Chitinase activity in the pooled fraction was 52.1 nmoles/min/ml giving a specific activity of 76.8 nmoles/min/mg of protein. This procedure yielded 24.4 mg of protein, which represented 35.2 % of the chitinase activity in the start material, resulting in a purification factor of 2.7 (Table 11).

Table 11 - Summary of the purification of recombinant chitinase by chromatography.

Sample	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Chitinase activity (nmoles/min/ml)	Specific activity (nmoles/min/mg)	% Yield of chitinase activity	Purification Factor
Soluble protein extract	15	12.4	186	354.8	28.6	100	0
After buffer-exchange Into 3M NaCl	21	9.5	199	318.8	33.6	126	-
Precipitate after Buffer-exchange	1	8.2	8.2	68.68	8.4	1.3	-
Pooled fractions from hydrophobic interaction chromatography	36	0.679	24.4	52.08	76.8	35.2	2.7
Pooled fractions from Anion-exchange chromatography	4	0.755	3.0	56.8	75.7	4.3	2.6
Pooled fractions from Gel filtration chromatography	30	0.060	1.8	6.64	110	3.7	3.8

Chitinase activity was measured using 4-Nitrophenyl-β-D-*N,N'*-Diacetylchitobiose. The percentage yield of chitinase activity was calculated relative to that in the soluble protein extract. The purification factor was calculated according to the relative increase in the specific activity of chitinase at each step of purification.

Anion-exchange Chromatography

Pooled fractions from HIC were purified further by anion-exchange chromatography on a Pharmacia Mono Q column. Figure 19 shows the elution profile from the MonoQ column. The chitinase eluted at approximately 0.24 M NaCl in fraction 14. Analysis by SDS-PAGE (Figure 20) showed that the chitinase was eluted between fractions 13-17. Further analysis of the purity of the protein was performed using a Western blot immuno-stained with rabbit anti-*P. aeruginosa* 385 serum (Figure 11). The results showed that chitinase was present in fractions 13-17 and is highly antigenic. However, there was a second band detected at 48 kDa. Fractions 13-16 were pooled and gave a protein concentration of 0.755 mg/ml. Chitinase assay result on the pooled sample gave 56.8 nmoles/min/ml activity which equated to a specific activity of 75.7 nmoles/min/mg. This is lower than that of the pooled sample from hydrophobic interaction chromatography. This procedure yielded 3 mg of protein, which represented 4.3 % of the chitinase activity in the soluble protein extract and gave a purification factor of 2.6. This demonstrates that anion-exchange chromatography did not result in any improvement in the purity of the chitinase obtained after HIC. Fractions 13-16 were pooled and purified further using gel filtration chromatography.

Gel Filtration Chromatography

500 µl of chitinase obtained from anion-exchange chromatography was purified further by gel filtration chromatography on a Pharmacia Superdex 200 column (Figure 21). A chitinase assay was performed on fractions 34-45, and activity was detected in fractions 34-42. Fractions 36-42, containing the majority of the activity, were pooled. A protein assay was performed which showed that the pooled sample was 60 µg/ml. Chitinase activity of the pooled sample was 6.64 nmoles/min/ml which had a specific activity of 110 nmoles/min/mg of protein. This procedure yielded 1.8 mg of protein, which represented 3.7 % of the chitinase activity in the soluble protein extract and gave a purification factor of 3.8.

Table 11 shows a summary of the specific activity of chitinase at each stage of chromatography purification. SDS-PAGE and Western blot analysis of all the samples in the chromatography purification can be seen in Figures 22 and 23, respectively. The Western blot shows that there is little difference between the HIC and Mono Q samples, as they both have many antigenic impurities. The greatest difference can be seen between the Mono Q sample and the gel filtration sample which is antigenically pure.

pH Activity Profile and Stability

The activity of chitinase, purified by chitin binding, as a function of pH was studied using 4-Nitrophenyl-β-D-*N*,*N*'-Diacetylchitobiose as a substrate and is shown in Figure 24. The graph shows that maximum activity was attained at pH 6.95, with over 90 % activity retained in the range pH 6.6-7.2 and 50 % in the range pH 6.1-7.8. Below pH 6.0 and above pH 8 activity fell rapidly. The results show a neutral

pH optimum unlike many other chitinases derived from many fungi and plants which show acidic pH optimum.

The pH stability of the recombinant chitinase was determined in the range pH 3-10 after incubation at 37 °C for 3 h. Residual activity was determined using 4-Nitrophenyl-β-D-*N,N'*-Diacetylchitobiose. Table 3 shows that the enzyme was inactive at pH 3.0. At pH 4.0 the activity was reduced by 58 %. Throughout the range pH 5-10 the enzyme was stable under the conditions employed.

Substrate Specificity

The recombinant chitinase showed activity against ethylene glycol chitin, chitin azure, colloidal chitin and a range of synthetic substrates. Table 4 shows the activity against a range of chitin substrates: the chitinase was active against all substrates tested. Table 5 shows the activity against a range of methlyumbelliferyl β-1, 4 linked glucosamine oligosaccharides. There was no activity against 4-Methylumbelliferyl *N*-Acetyl-β-D-Glucosaminide indicating a lack of exochitinase (β-1,4 -hexosaminidase) activity. Activity was measured against 4-Methylumbelliferyl-β-D-*N,N'*-Diacetylchitobioside, 4-Methylumbelliferyl-β-D-*N,N',N''*-Triacetylchitotrioside , and 4-Methylumbelliferyl-β-D-*N,N',N'',N'''*-Tetraacetylchitotetraoside. These all represent oligosaccharide analogues of two or more sugars and demonstrate the endo-splitting nature of the chitinase activity. The highest activity was against 4-Methylumbelliferyl-β-D-*N,N'*-Diacetylchitobioside which suggests that the primary function of the enzyme is the removal of diacetylchitobiose from the non-reducing end of chitin or chitodextrins.

Many chitinases display lysozyme activity and are able to depolymerise the peptidoglycan of bacterial cell walls by the hydrolysis of β-1,4 linkages between *N*-

acetylmuramic acid and *N*-acetyl-D-glucosamine. However, the chitinase from *Pseudomonas aeruginosa* 385 did not display any lysozyme activity as determined by the lack of lysis of *Microccocus lysodeikticus* cells.

Kinetic Studies

The K_m, k_{cat} and specificity constant (k_{cat}/K_m) were calculated for the substrates 4-Nitrophenyl-β-D-*N*,*N'*-Diacetylchitobiose and 4-Nitrophenyl-β-D-*N*,*N'*,*N''*-Triacetylchitotriose and are shown in Table 6. The specificity constants for both substrates are similar, but the K_m's and k_{cat}'s differ by an order of magnitude. The lowest K_m being that for 4-Nitrophenyl-β-D-*N*,*N'*,*N''*-Triacetylchitotriose and the highest turnover number for 4-Nitrophenyl-β-D-*N*,*N'*-Diacetylchitobiose. Although the specificity constants are comparable, indicating similar substrate preference, the kinetic data suggest the enzyme may have evolved to maximise rates against the removal of diacetylchitobiose from the non-reducing end of chitin or chitodextrins by increasing both K_m and k_{cat}.

Binding Studies

Table 7 shows the results of investigations into the binding of recombinant chitinase to a number of different insoluble carbohydrate substrates. Over 90% of the chitinase was bound to three of the chitin substrates, however only 17% bound to chitosan. Over 50% of the chitinase bound to lichenan and microgranular cellulose but less than 40% bound to xylan, heparin agarose and *N,N'*-Diacetylchitobiose agarose.

Example 6 - Cloning and Expression of the *gro*EL gene from *Pseudomonas aeruginosa* Isolate 385

Bacterial Strain and Growth of Cultures

Pseudomonas aeruginosa isolate 385 was obtained and grown as described in Example 4.

Recombinant *Escherichia coli* strains were grown as described in Example 5.

Standard Molecular Biology Techniques

Standard molecular biology techniques were as described in Example 5.

Extraction of Genomic DNA, Extraction of Plasmid DNA, Oligonucleotide Synthesis, Nucleotide Sequencing and Sequence Analysis, Polymerase Chain Reaction (PCR)

These were as described in Example 5.

Cloning of the *gro*EL Gene of *P. aeruginosa* Isolate 385

The nucleotide sequence of the *gro*EL gene (encoding Hsp60 ; heat shock protein 60) of *Pseudomonas aeruginosa* strain PA01 has been disclosed - GenBank accession number U17072. Two further *P. aeruginosa gro*EL sequences were found in public databases, these being accession numbers S77424 (strain P1118, O:3) and M63957 (strain ATCC27853). Each of these accessions includes additional sequence, both 5' and 3' to the *gro*EL coding region. The three sequences above

were aligned using DNASIS v2.5 software and regions were identified for the design of primers that would facilitate amplification by PCR of the *gro*EL gene with 5' and 3' flanking DNA. These primers had the sequence 5'-CTTACTCCGGCAGCAAC-3' and 5'-GACCTGAAGCAGGCACTG-3'. A PCR product was obtained using these primers, with genomic DNA from *P. aeruginosa* 385 being used as template. This product was sequenced in order to determine the 5' and 3' ends of the *gro*EL gene of isolate 385. The information obtained was used to design primers that could be used in PCR to amplify the coding region of the isolate 385 *gro*EL gene. These primers had the following sequences ; 5'-ATGGCTGCCAAAGAAGTTAAG-3' and 5'-TTACATCATGCCGCCCATG-3'. A PCR product was obtained with these primers, employing Pfu DNA polymerase and the following thermal cycling program; 30 cycles of, denaturing at 94 °C for 45 s, annealing at 52 °C for 1 min and extension at 72 °C for 4.5 min. This product was phosphorylated with T4 polynucleotide kinase and cloned into the pTrcHisB expression vector (Invitrogen), prepared as follows : pTrcHisB vector was restricted with *Bam*HI and the resulting 5' overhangs were removed by treatment with Mung bean nuclease (New England Biolabs). The vector was then dephosphorylated by calf intestinal alkaline phosphatase to prevent self-ligation. The pTrcHisB vector facilitates the expression of a fusion polypeptide that includes tags for purification and detection of the recombinant protein. The purification tag consists of a hexahistidine sequence that allows affinity purification on immobilised metal ion matrices. The detection tag is a short sequence for which a specific antibody is available (Invitrogen Anti-Xpress antibody) and this overlaps with an enterokinase recognition site that allows removal of the tags from the fusion protein.

The pTrcHisB vector containing the *gro*EL gene was transformed into *E. coli* TOP10 cells and positive clones were identified by PCR analysis of extracted

plasmids. One clone was selected for use in expression experiments and for DNA sequencing.

Production of Recombinant GroEL Protein

A glycerol stock of *E. coli* TOP10 cells carrying the pTrcHisB vector with the cloned *gro*EL gene was streaked onto an LB medium agar plate containing ampicillin. The plate was incubated at 37 °C for 16-18 h and a single colony used to inoculate 5 ml LB medium supplemented with ampicillin. This starter culture was grown at 37 °C, with shaking at 200 rpm, for 16-18 h. This culture was used to inoculate three 2-litre flasks, each containing 600 ml LB medium, with ampicillin, and these incubated as above. When the optical density (O.D.) at 600 nm of the cultures was approximately 0.7 (t=0), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and growth continued for 4 h. Cells were harvested in 50 ml aliquots by centrifugation at 3500 x *g* for 15 min, the supernatant removed and the cell pellet stored at –20 °C.

Purification of Recombinant GroEL

The recombinant GroEL fusion protein obtained above was found to be present in inclusion bodies. This allowed an extraction to be performed using B-PER extraction reagent (Pierce Chemical Co.) which solubilises most cell proteins but does not solubilise inclusion body proteins.

Four cell pellets from above were thawed at room temperature and each resuspended with 5 ml of B-PER extraction reagent by vortexing for 3 min. Insoluble material, including inclusion bodies, was collected by centrifugation at 12000 x *g* for 15 min. The supernatants were removed and retained (B-PER soluble proteins) and the

pellets were re-extracted with a further 5 ml of B-PER, as above. Recombinant GroEL was purified from the resulting insoluble material using Clontech TALONspin columns. These columns contain the TALON resin, which incorporates immobilised cobalt ions for coordination of the hexahistidine tag sequence. The purification was carried out according to the manufacturer's instructions. Briefly, each pellet obtained above was solubilised by shaking (150 rpm) at room temperature for 20 min with 1 ml of lysis buffer (50 mM NaH_2PO_4, 10 mM Tris-HCl, 6 M Guanidinium-HCl, 100 mM NaCl pH 8.0). This buffer should solubilise inclusion body protein and any insoluble material was removed by centrifugation at 16000 x *g* for 10 min. The solubilised protein from one pellet was allowed to bind to the resin of one TALONspin column by incubation on a roller table for 10 min. Unbound material was removed by centrifugation at 700 x *g* for 2.5 min. The resin was washed three times with 1 ml of wash buffer (50 mM NaH_2PO_4, 100 mM NaCl, 6 M Guanidinium-HCl pH 7.0). The bound protein was then eluted with 2 x 0.5 ml aliquots of elution buffer (50 mM NaH_2PO_4, 20 mM Piperazine, *N,N'*-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 6 M Guanidinium-HCl pH 6.0). Aliquots taken throughout the procedure were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Eluates from the four columns were combined and then buffer-exchanged into 50 mM sodium phosphate buffer pH 7.0, using a Pharmacia PD10 column (see below). A protein assay was performed (see below) and the sample concentrated to approximately 1 mg/ml using a Vivaspin 15 ml ultrafiltration tube (5 kDa molecular weight cut-off membrane), according to the manufacturer's recommendations. The protein concentration of the sample was then re-assayed.

Buffer-exchange of Samples

This was carried out as described in Example 4.

Protein Assay

Protein concentrations were determined using a Pierce BCA protein assay kit. Assays were performed according to the manufacturer's instructions, with bovine serum albumin (BSA) being used to construct a standard curve. The absorbance at 570 nm of the samples and standards was measured on a Labsystems iEMS MF plate reader and the protein concentration calculated using the Labsystems Genesis software.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Samples containing recombinant GroEL protein were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described in Example 1 except as follows.

Samples containing guanidinium salts were precipitated for 10 min at -20 °C, following addition of ice-cold ethanol to a final concentration of 90 % (v/v). The precipitate was collected by centrifugation at 16000 x *g* for 5 min and then washed with one volume of ice-cold 90 % (v/v) ethanol (equivalent volume to that added for precipitation). The pellet was air-dried for 5 min then resuspended with 1 x SDS-PAGE sample buffer.

For analysis of whole-cell lysates of cells expressing recombinant GroEL, 1 ml of culture was centrifuged at 16000 x *g* for 2 min. The supernatant was removed and the cell pellet resuspended in 50 µl of 1 x SDS-PAGE sample buffer.

Samples and standards were heated at 100 °C for 4 min and 10 µl of each was loaded into a well of the gel. For whole-cell lysate samples, 8 µl was loaded for the sample taken prior to addition of IPTG (t=0). A proportionately lower volume of the t=4h sample was loaded, according to the increase in O.D. at 600 nm. Electrophoresis was performed according to the Bio-Rad procedure at a constant 200 V, until the dye front had reached the bottom of the gel (approximately 40 minutes). After electrophoresis, gels were stained with 0.1 % (w/v) Coomassie Brilliant Blue R-250 in 30 % (v/v) methanol and 10 % (v/v) acetic acid. Proteins were visualised by destaining gels in 30 % (v/v) methanol and 10 % (v/v) acetic acid.

Western Blotting and Immunostaining

This was carried out as described in Example 2.

Results

Cloning and Expression of the *gro*EL Gene

The sequence in Figure 25 shows the data obtained for the *gro*EL gene and flanking DNA in isolate 385. This data is derived from sequencing of both the cloned *gro*EL gene and the PCR product of the *gro*EL gene that was obtained with flanking DNA. The coding region of the gene is 98.6-99.7 % identical to the *gro*EL gene sequences of *P. aeruginosa* strains P1118 O:3 (accession S77424) and ATCC27853 (accession M63957). The corresponding protein sequences are 98.1-99.8 % identical. The 3' end of the isolate 385 gene incorporates four copies of a nine base pair sequence (ATGGGCGGC), located at positions 1691-1699, 1709-1717, 1718-1726 and 1727-1735 in the sequence of Figure 25. The cloned product, which corresponds to the only the coding region of the gene, lacked the final copy of this repeat, presumably

because of binding of the 3' primer at the wrong location. The 3' primer binds to a region that includes one full copy of the repeated sequence and this leads to the possibility of mispriming away from the most 3' repeat. It was concluded that the reverse primer bound to nucleotides 1717-1735 instead of nucleotides 1726-1744, as numbered in Figure 25. Binding at this site would still allow extension by DNA polymerase despite the mismatches between the 5' end of the primer and this site. This eventuality could not be avoided without designing an impractically long 3' primer. The mispriming of the PCR leads to the lack of three amino acid residues at the C-terminus of the protein, with respect to the native protein. This difference is not expected to alter significantly the immunogenicity of the protein. The sequenced clone also lacks three bases of the vector that occur at the splice site with the 5' end of the *gro*EL gene, presumably arising from nuclease action occurring during preparation of the vector. This leads to a lack of one amino acid residue that is encoded by the vector but retains the correct reading frame of the cloned *gro*EL gene. The DNA sequence and predicted amino acid sequence of the fusion gene obtained are shown in Figure 26. This yields a polypeptide of 574 amino acid residues with a predicted molecular weight of 60.1 kDa.

When recombinant protein expression was induced in cells containing the pTrcHisB-groEL plasmid, a protein with an apparent molecular weight of 64 kDa was seen to accumulate to a very high proportion of total cell protein. This was assessed by SDS-PAGE analysis of whole cell lysates of cells taken from the expression culture. This protein is of a similar molecular weight to that predicted for the GroEL fusion protein. When cells were extracted with B-PER reagent, this protein was seen to be located in the B-PER insoluble fraction, implying that it is present within inclusion bodies.

Purification of Recombinant GroEL Fusion Protein

Figure 27 shows a Coomassie stained SDS gel of fractions obtained from the purification procedure outlined above. The recombinant GroEL fusion protein is located almost exclusively in the B-PER insoluble fraction following cell extraction. A large proportion of this protein does not bind to the TALONspin column and small amounts are eluted during the three wash steps. Most of the contaminating proteins do not bind to the resin and those that do are largely removed at the wash steps. The protein eluted from the resin is estimated to be greater than 99 % pure, as judged from the stained gel. Samples taken throughout the purification procedure were transferred to a membrane by Western blotting and the blot was immuno-stained with anti-*P. aeruginosa* 385 serum (Figure 28). The GroEL fusion protein was detected by the antiserum and showed a strong antigenic response at 64kDa. The purified protein contains one other species that reacts with the antiserum, this being at 32 kDa. This band is not visible on the Coomassie stained SDS gel (Figure 27) and is presumed to be only a minor component of the sample. The protein concentration of the eluate, after buffer-exchange into 50 mM sodium phosphate buffer pH 7.0 was determined as 216 μg/ml, with a total volume of 7 ml. The volume of this sample was reduced from 7 ml to 1 ml using an ultrafiltration membrane. The protein concentration of the retentate was determined to be 0.6 mg/ml, suggesting that there was a 60% loss of protein during this step.

CLAIMS

1. A protein from *P. aeruginosa* which has the following N-terminal sequence:

I A-A-K-E-(M or V)-K-F-S;

II (Q,M or V)-A-R-E-D-A-A-A-A-M;

III M-(I or L)-R-I-D-(F and/or Q); or

IV M-(L or I)-R-I-D.

2. A protein having an amino acid sequence as shown in Figure 8, wherein N terminal sequence of the protein is either M-I-R-I-D or Q-A-R-E-D-A-A-A-A-M.

3. A protein comprising an amino acid sequence as shown in Figure 25 or Figure 26.

4. A protein which is a homologue or derivative of a protein as defined in any one of claims 1 to 3.

5. A protein as claimed in any preceding claim provided in substantially pure form.

6. An antigenic and/or immunogenic fragment of a protein as claimed in any preceding claim.

7. A fragment as claimed in claim 6, comprising the sequence:

I A-A-K-E-(M or V)-K-F-S;

II (Q,M or V)-A-R-E-D-A-A-A-A-M;

III M-(I or L)-R-I-D-(F and/or Q); or

IV M-(L or I)-R-I-D.

8. An antigen composition comprising one or more proteins as claimed in any one of claims 1 to 5, and/or one or more fragments as claimed in claim 6 or claim 7.

9. An antigen composition as claimed in claim 8, which further comprises one or more other *P. aeruginosa* antigens and/or immunogens.

10. A protein as claimed in any one of claims 1 to 5, a fragment as claimed in claim 6 or claim 7, or an antigen composition as claimed in claim 8 or claim 9 for use in the detection of *P. aeruginosa*.

11. A method of detecting and/or diagnosing *P. aeruginosa* which comprises:

(a) bringing into contact with a sample to be tested a protein as claimed in any one of claims 1 to 5, a fragment as claimed in claim 6 or claim 7, or an antigen composition as claimed in claim 8 or claim 9; and

(b) detecting the presence of antibodies to *P. aeruginosa*.

12. A method as claimed in claim 11, wherein the sample is a sample of mucous or saliva.

13. An antibody capable of binding to a protein as defined in any one of claims 1 to 5.

14. A method for the detection/diagnosis of *P. aeruginosa* which comprises the step of bringing into contact a sample to be tested and an antibody as defined in claim 13.

15. The use of a protein as claimed in any one of claims 1 to 5, a fragment as claimed in claim 6 or claim 7, an antigenic composition as claimed in claim 8 or claim 9, or an antibody as claimed in claim 13 in detecting and/or diagnosing *P. aeruginosa*.

16. A method as claimed in claim 11 or claim 12 or the use as claimed in claim 15, wherein the detecting and/or diagnosing is carried out *in vitro*.

17. A kit for use in the detection and/or diagnosis of *P. aeruginosa*, which kit comprises a protein as claimed in any one of claims 1 to 5, a fragment as claimed in claim 6 or claim 7, an antigen composition as claimed in claim 8 or claim 9, or an antibody as claimed in claim 13.

18. The use of a protein as claimed in any one of claims 1 to 5, a fragment as claimed in claim 6 or claim 7, an antigenic composition as claimed in claim 8 or claim 9, or an antibody as claimed in claim 13 in medicine.

19. A composition capable of eliciting an immune response in a subject, which composition comprises a protein as claimed in any one of claims 1 to 5, a fragment as claimed in claim 6 or claim 7, or an antigen composition as claimed in claim 8 or claim 9.

20. A composition as claimed in claim 19 which is a vaccine composition, optionally further comprising one or more adjuvants.

21. The use of a protein as claimed in any one of claims 1 to 5, a fragment as claimed in claim 6 or claim 7, or an antigen composition as claimed in claim 8 or claim 9 in the preparation of an immunogenic composition, preferably a vaccine.

22. The use of an immunogenic composition as claimed in claim 21 in inducing an immune response in a subject.

23. A method for the treatment or prophylaxis of *P. aeruginosa* infection in a subject, which comprises the step of administering to the subject an effective amount of a protein as claimed in any one of claims 1 to 5, a fragment as claimed in claim 6 or claim 7, or an antigen composition as claimed in claim 8 or claim 9.

24. A method as claimed in claim 23, wherein the subject is suffering from cystic fibrosis.

25. A method as claimed in claim 23 or claim 24, wherein the protein, fragment, or antigen composition is administered in the form of a vaccine.

26. A nucleic acid molecule comprising or consisting of a sequence which is:

(i) the DNA sequence set out in Figure 8, 14, 25 or 26 or its RNA equivalent;

(ii) a sequence which is complementary to the sequence of (i);

(iii) a sequence which codes for the same protein or polypeptide, as the sequence of (i) or (ii);

(iv) a sequence which is has substantial identity with any of those of (i), (ii) and (iii);

(v) a sequence which codes for a homologue, derivative or fragment of a protein as defined in Figure 8, 14, 25 or 26.

27. A vector comprising a nucleic acid molecule as defined in claim 26.

28. A host cell transformed with a vector as defined in claim 27.

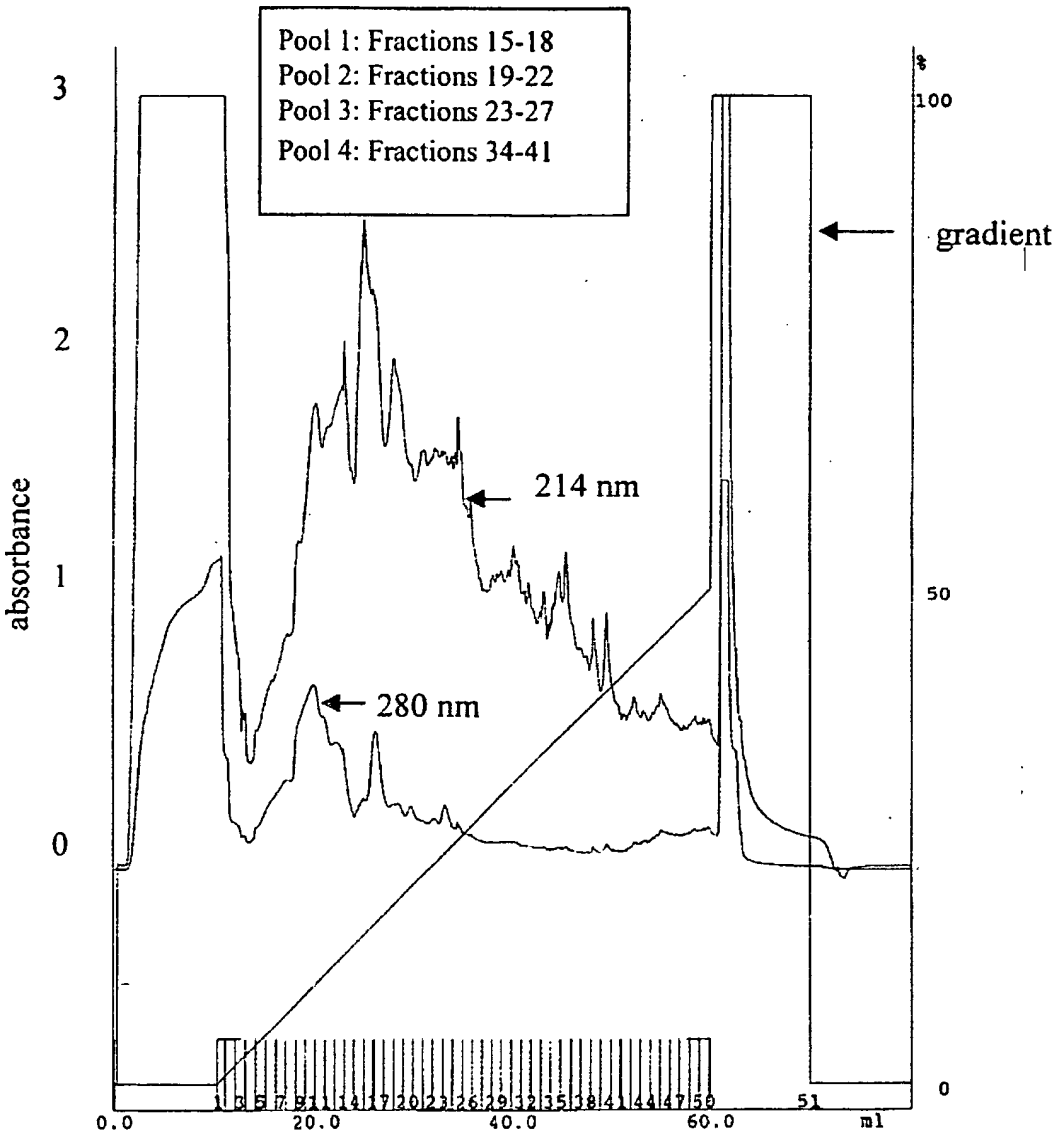
29. A vaccine composition comprising one or more nucleic acid molecules as defined in claim 26.

30. A method for the detection/diagnosis of *P. aeruginosa* which comprises the step of bringing into contact a sample to be tested and at least one nucleic acid as defined in claim 29.

31. A method of determining whether a protein as defined in any one of claims 1 to 5 represents a potential antimicrobial target, which method comprises inactivating said protein and determining whether *P. aeruginosa* is still viable *in vitro* or *in vivo*.

32. The use of an agent capable of antagonsing, inhibiting or otherwise interfering with the function or expression of a protein as defined in any one of claims 1 to 5 in the manufacture of a medicament for the treatment or prophylaxis of *P. aeruginosa* infection.

Figure 1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Pool 3: Fractions 23-27
Pool 4: Fractions 34-41
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1
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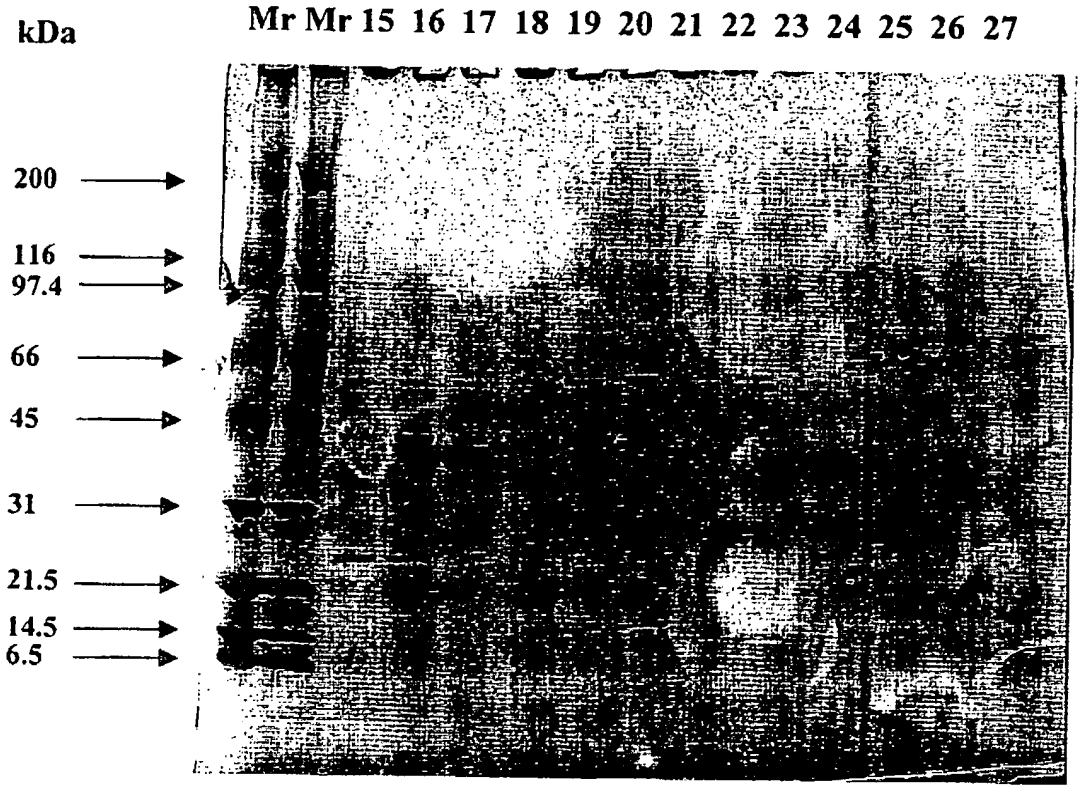
214 nm
280 nm
gradient
%
100
50
0
0.0
20.0
40.0
60.0
51
ml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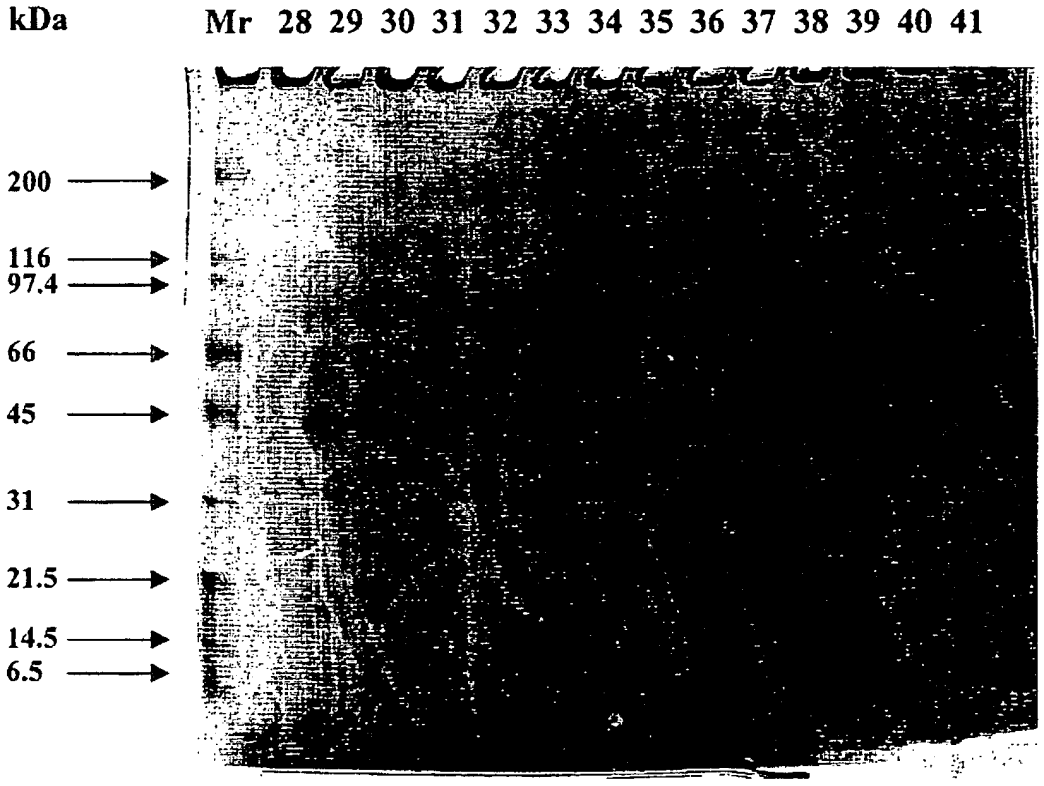
66
45
31
21.5
14.5
6.5

Figure 2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200
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97.4
66
45
31
21.5
14.5
6.5

Figure 3

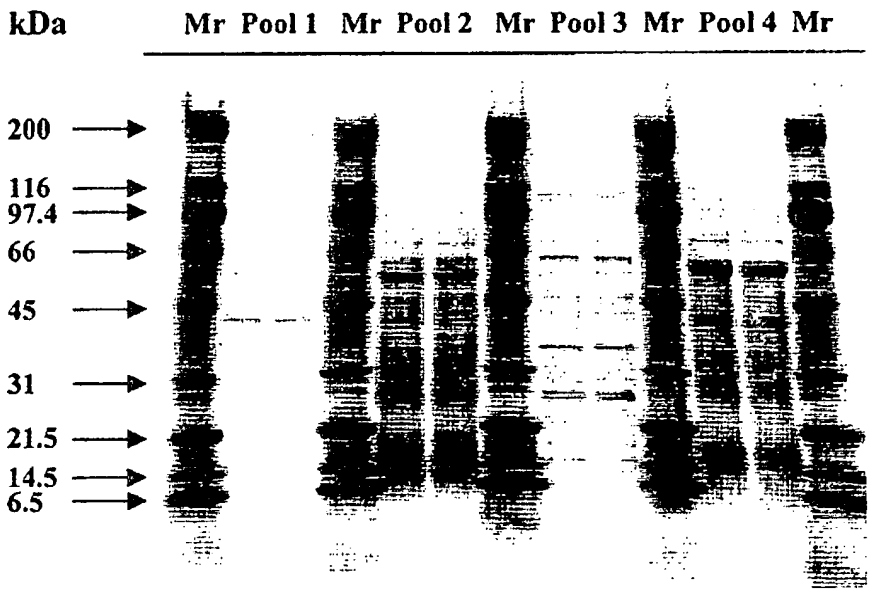

kDa
Mr
Pool 1
Mr
Pool 2
Mr
Pool 3
Mr
Pool 4
Mr
200
116
97.4
66
45
31
21.5
14.5
6.5

Figure 4

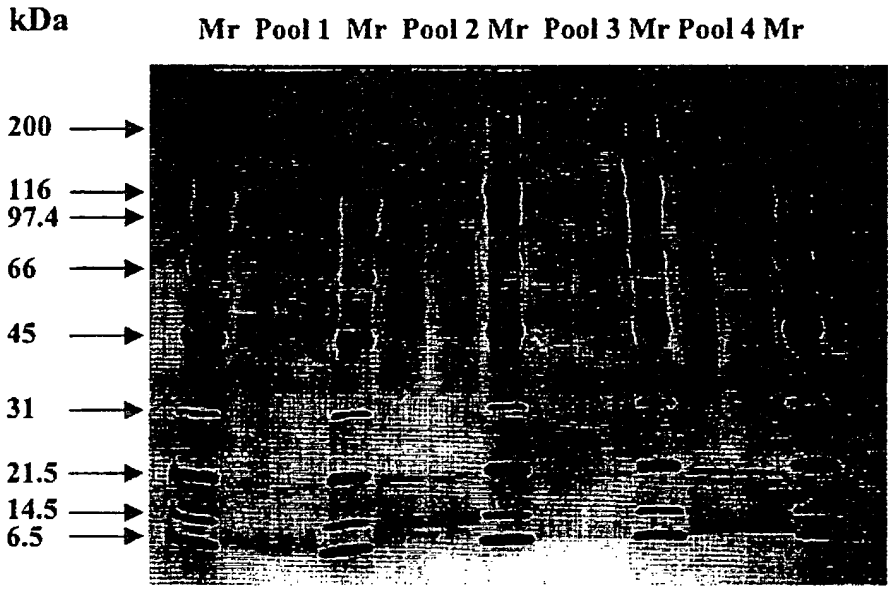

kDa
Mr Pool 1 Mr Pool 2 Mr Pool 3 Mr Pool 4 Mr
200
116
97.4
66
45
31
21.5
14.5
6.5

Figure 5

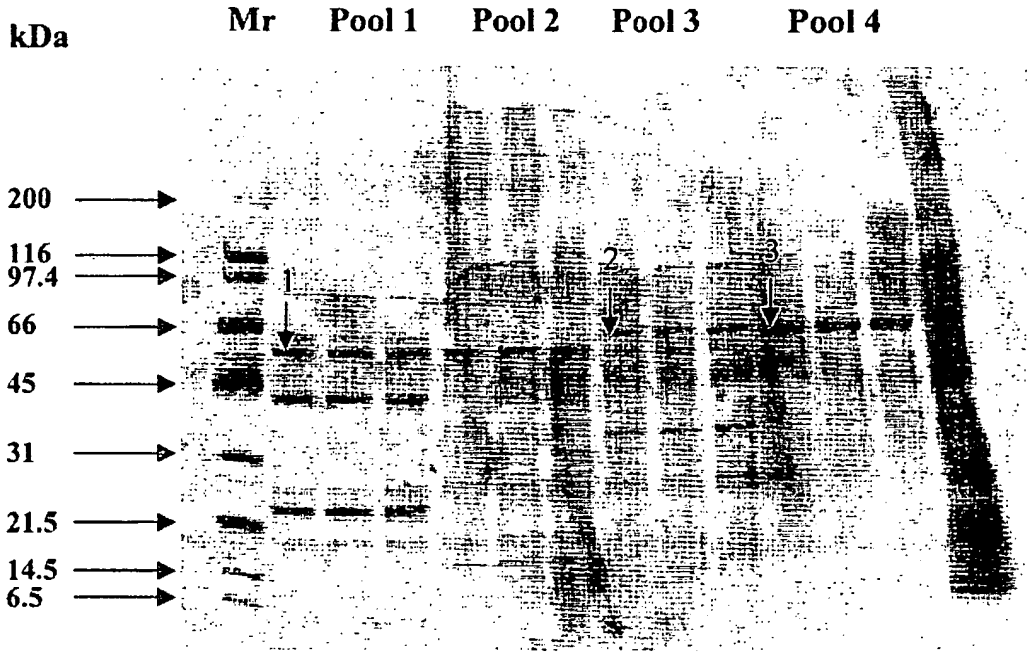

kDa
Mr
Pool 1
Pool 2
Pool 3
Pool 4
200
116
97.4
66
45
31
21.5
14.5
6.5
1
2
3

Figure 6

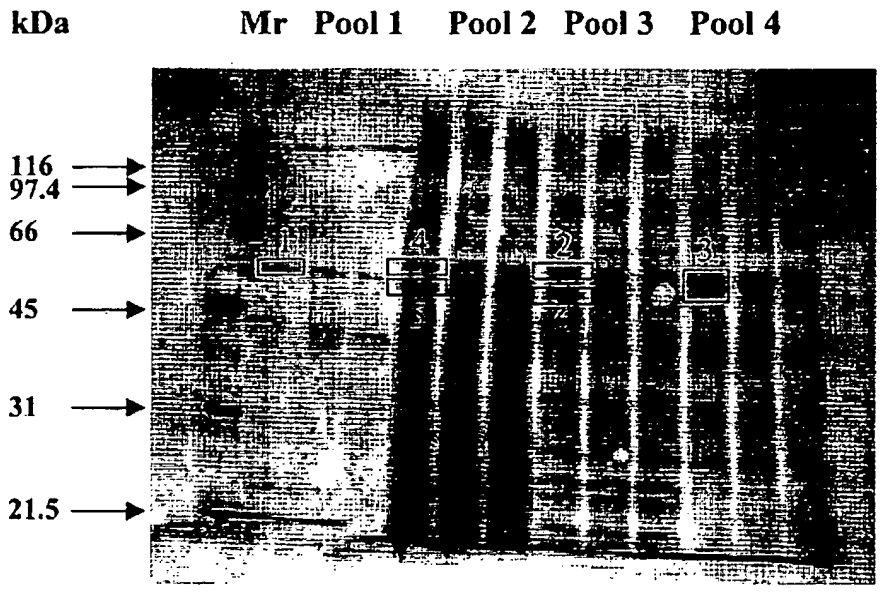


Figure 7

Figure 8

```
                9         18        27        36        45        54
5'  ATG ATC AGG ATC GAC TTT TCC CAG TTG CAC CAG GCC CGC GAA GAT GCC GCG GCG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     M   I   R   I   D   F   S   Q   L   H   Q   A   R   E   D   A   A   A

               63        72        81        90        99       108
    GCC ATG CCG AGC ATC GCC GGC AAG AAG ATT CTC ATG GGC TTC TGG CAC AAC TGG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     A   M   P   S   I   A   G   K   K   I   L   M   G   F   W   H   N   W

              117       126       135       144       153       162
    CCG GCC GGC GCC GCC GAC GGC TAC CAG CAG GGC TCG TTC GCC AAC ATC GCG CTG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     P   A   G   A   A   D   G   Y   Q   Q   G   S   F   A   N   I   A   L

              171       180       189       198       207       216
    GAA GAC GTG CCG AGC GAG TAC AAC GTG GTC GCC GTG GCC TTC ATG AAA GGG CGC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     E   D   V   P   S   E   Y   N   V   V   A   V   A   F   M   K   G   R

              225       234       243       252       261       270
    GGC ATC CCG ACC TTC CAG CCA TAC AAC CTG TCC GAC GCG GAG TTT CGC CGC CAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     G   I   P   T   F   Q   P   Y   N   L   S   D   A   E   F   R   R   Q

              279       288       297       306       315       324
    GTC GGC GTG CTC AAC GCC CAG GGC CGC GCG GTG CTG ATT TCG CTG GGG GGC GCC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     V   G   V   L   N   A   Q   G   R   A   V   L   I   S   L   G   G   A

              333       342       351       360       369       378
    GAC GCG CAC ATC GAG TTG CAC GCC GGG CAG GAG CAG GCG CTG GCC GCC GAG ATC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     D   A   H   I   E   L   H   A   G   Q   E   Q   A   L   A   A   E   I

              387       396       405       414       423       432
    GTC CGT CTG GTG GAA ACC TAC GGT TTC GAC GGC CTG GAC ATC GAC CTC GAG CAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     V   R   L   V   E   T   Y   G   F   D   G   L   D   I   D   L   E   Q

              441       450       459       468       477       486
    AGC GCC ATC GAC CTG GCC GAC AAC CAG CGG GTG CTG CCG GCG GCC CTC AAG CTG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     S   A   I   D   L   A   D   N   Q   R   V   L   P   A   A   L   K   L

              495       504       513       522       531       540
    GTG CGC GAG CAC TAC GCC GGG CAG GGC AAG CAC TTC ATC GTC AGC ATG GCC CCG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     V   R   E   H   Y   A   G   Q   G   K   H   F   I   V   S   M   A   P

              549       558       567       576       585       594
    GAG TTT CCC TAT CTG CAC AAG AAC GGC AAG TAC GTG CCT TAT CTG CAG GCC CTG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     E   F   P   Y   L   H   K   N   G   K   Y   V   P   Y   L   Q   A   L
```

Figure 8 continued

```
        603         612         621         630         639         648
GAA GGC GTC TAC GAC TTC ATC GCG CCG CAG TAC TAC AAC CAG GGC GGC GAC GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   G   V   Y   D   F   I   A   P   Q   Y   Y   N   Q   G   G   D   G

        657         666         675         684         693         702
CTG TGG GTC CAG GAG GCG AAC GGC GGC AAG GGC GCC TGG ATC GCG CAG AAC AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   W   V   Q   E   A   N   G   G   K   G   A   W   I   A   Q   N   N

        711         720         729         738         747         756
GAC GCG ATG AAA GAA GAC TTC CTC TAC TAC CTC ACC GAG AGC CTG GCC ACC GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   A   M   K   E   D   F   L   Y   Y   L   T   E   S   L   A   T   G

        765         774         783         792         801         810
AGC CGC GAC TTC GTG CGG ATC CCG GCG CAG CGC CTG GCC ATC GGC CTG CCG AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   R   D   F   V   R   I   P   A   Q   R   L   A   I   G   L   P   S

        819         828         837         846         855         864
AAC GTC GAT GCG GCG GCT ACC GGC TAC GTG ATC GAT CCC GCC GCG GTG AGC AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 N   V   D   A   A   A   T   G   Y   V   I   D   P   A   A   V   S   N

        873         882         891         900         909         918
GCG TTT CGT CGC CTG GAA GCC GCC GGT CAC GCG ATC AAG GGC CTG ATG ACC TGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 A   F   R   R   L   E   A   A   G   H   A   I   K   G   L   M   T   W

        927         936         945         954         963         972
TCG GTG AAC TGG GAC GAT GGC CTG AAC AAG CGC GGC GAG CGC TAC AAC TGG GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   V   N   W   D   D   G   L   N   K   R   G   E   R   Y   N   W   E

        981         990         999        1008        1017        1026
TTC CGC AAG CGC TAC GCC AGC CTC ATC CAT GAC GGC GAG GGC GGC GAC CAG CGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 F   R   K   R   Y   A   S   L   I   H   D   G   E   G   G   D   Q   R

       1035        1044        1053        1062        1071        1080
CCG GCG GCG CCG CAG GGC CTG CGT CTG CTG GAG CGC GGC GAG ACC AGC CTG GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   A   A   P   Q   G   L   R   L   L   E   R   G   E   T   S   L   V

       1089        1098        1107        1116        1125        1134
CTG GCC TGG AAC GCC TCC AGC GGG CAG CGT CCG ATC GAT TAC TAC AGC CTC TAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   A   W   N   A   S   S   G   Q   R   P   I   D   Y   Y   S   L   Y

       1143        1152        1161        1170        1179        1188
CGC GAC GGC GCC ATG GTT GGC CAG AGC GCC GCG CTG GGT TCC ACC GAC AGC GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   D   G   A   M   V   G   Q   S   A   A   L   G   S   T   D   S   G
```

Figure 8 continued

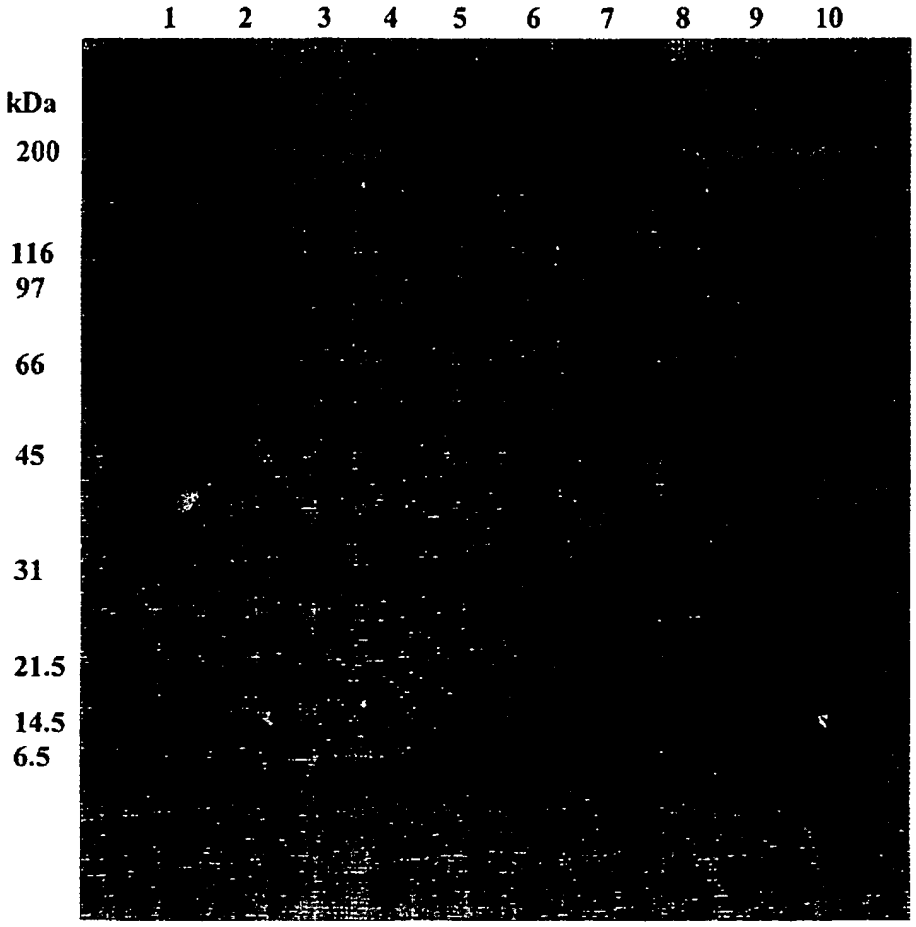
```
      1197        1206        1215        1224        1233        1242
CTG ACG GCG GAC ACC CGC TAC AGC TAT TTC GTC ACC GCC ACC GAT ACC CAG GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   T   A   D   T   R   Y   S   Y   F   V   T   A   T   D   T   Q   G

      1251        1260        1269        1278        1287        1296
AAC CAG TCG CTG CCC AGC GAA GGC CTG GAG GTC AGC ACC AGC GGC GGG GCG GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 N   Q   S   L   P   S   E   G   L   E   V   S   T   S   G   G   A   V

      1305        1314        1323        1332        1341        1350
GAT CCG CAA TTC CCG CAA TGG CGG GAG AAC CAG GCC TAT CGG GTC GAC GAT GGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   P   Q   F   P   Q   W   R   E   N   Q   A   Y   R   V   D   D   G

      1359        1368        1377        1386        1395        1404
GTG ACC TAC GAG GGG CTG CGC TAT CTC TGC CTG CAG GCG CAC ACC TCC AAC AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   T   Y   E   G   L   R   Y   L   C   L   Q   A   H   T   S   N   S

      1413        1422        1431        1440        1449
GGC TGG ACG CCG CCG GTA GCC TTC ACC CTC TGG CGG CCG CTG CGC TGA 3'
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   W   T   P   P   V   A   F   T   L   W   R   P   L   R   *
```



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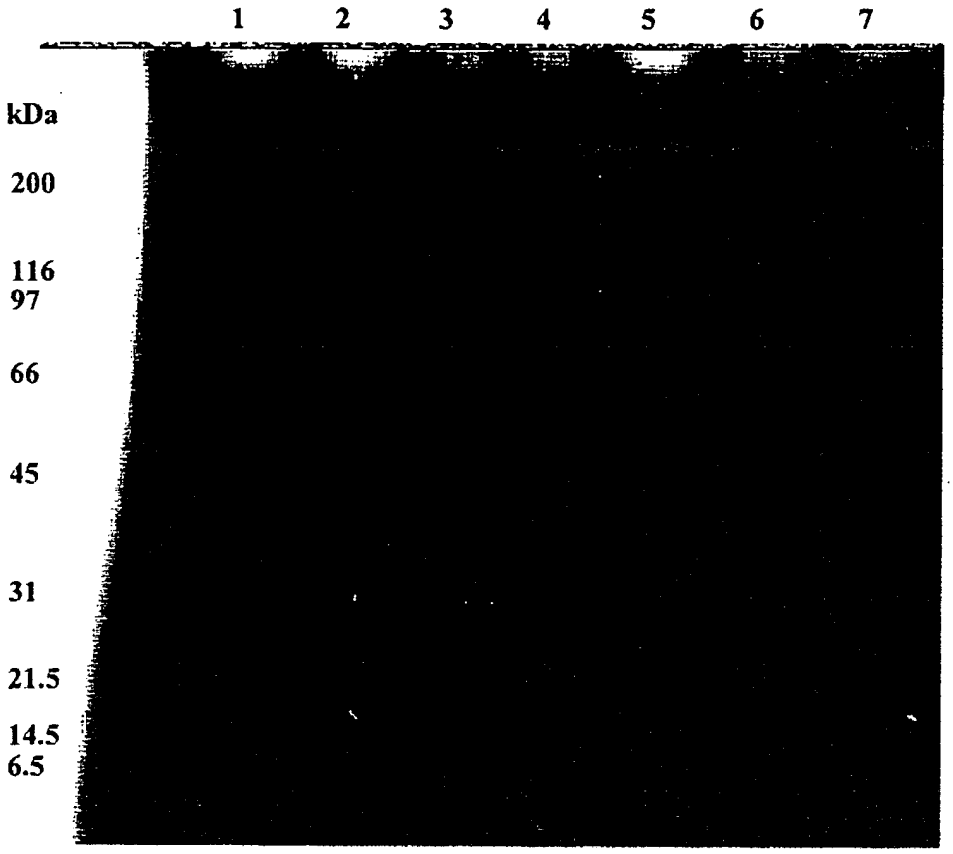
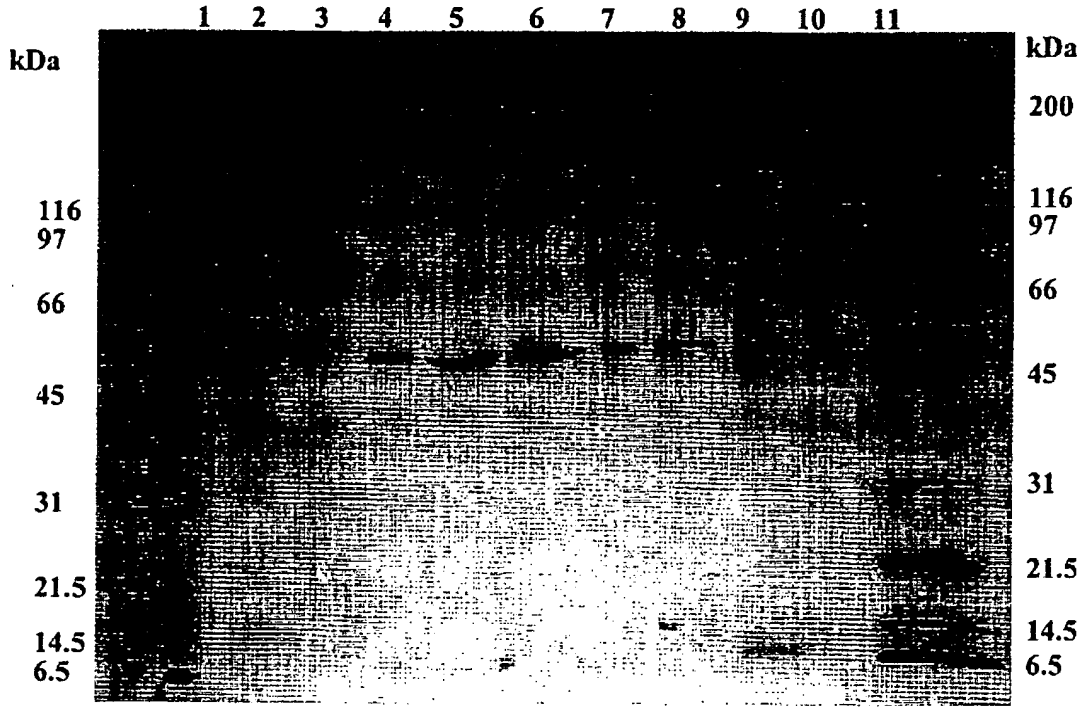


Figure 10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kDa
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97
66
45
31
21.5
14.5
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kDa
200
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97
66
45
31
21.5
14.5
6.5

Figure 11

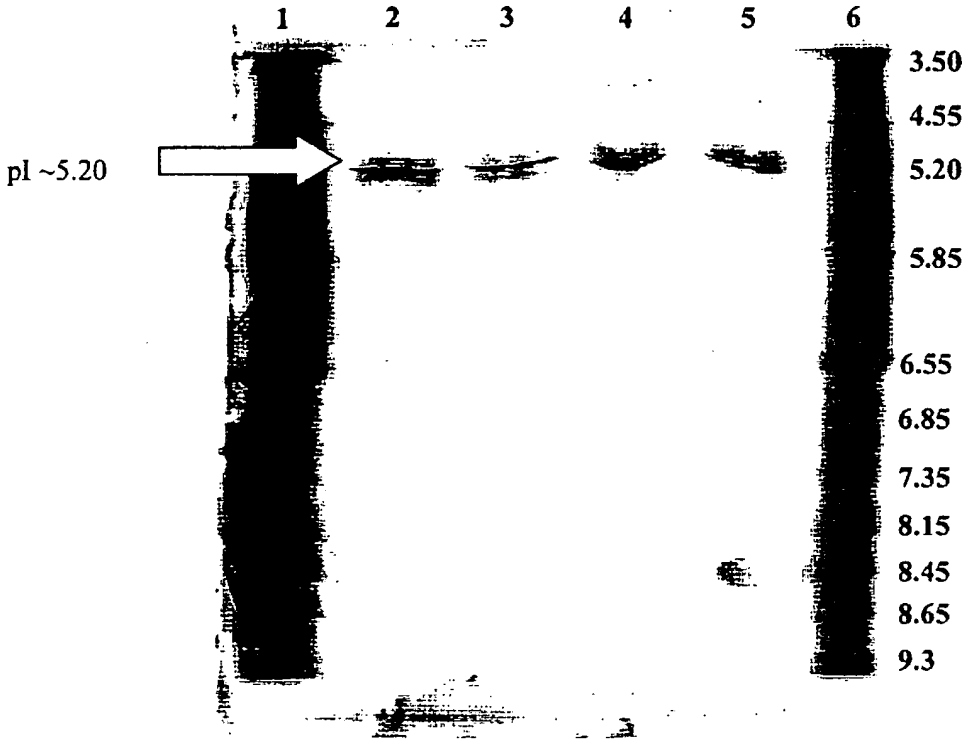

Figure 12

Figure 13

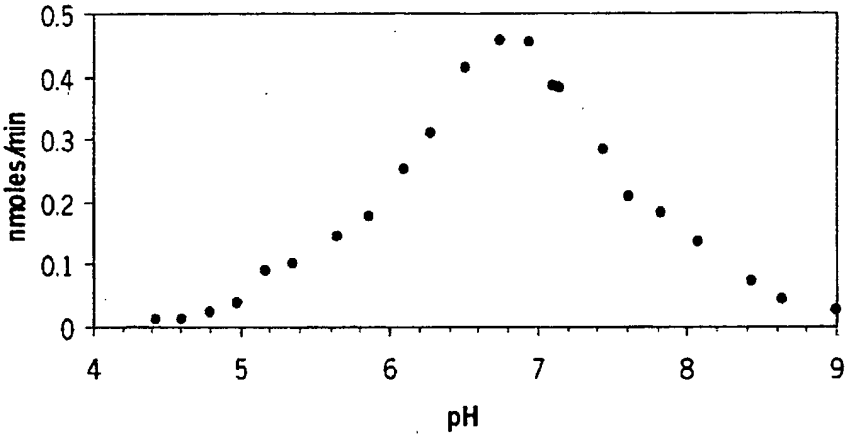

Figure 14

CGTCTCTGCAACCATGCCCCCGCCCTGATCGCGACAAACGCCGGAATGCCCCGTGGTAGACGCTCGCGCCTGTTTTT
TCTAAGCGTTCGCGGCGGCCACGGCGCGGTTCGTCGGACAGCCCCTCCGGATGCGTTTCCTACCTTCATCCGAGGAG
CGGTAA

```
ATG ATC AGG ATC GAC TTT TCC CAG TTG CAC CAG GCC CGC GAA GAT    205
 M   I   R   I   D   F   S   Q   L   H   Q   A   R   E   D     15

GCC GCG GCG GCC ATG CCG AGC ATC GCC GGC AAG AAG ATT CTC ATG    250
 A   A   A   A   M   P   S   I   A   G   K   K   I   L   M     30

GGC TTC TGG CAC AAC TGG CCG GCC GGC GCC GCC GAC GGC TAC CAG    295
 G   F   W   H   N   W   P   A   G   A   A   D   G   Y   Q     45

CAG GGC TCG TTC GCC AAC ATC GCG CTG GAA GAC GTG CCG AGC GAG    340
 Q   G   S   F   A   N   I   A   L   E   D   V   P   S   E     60

TAC AAC GTG GTC GCC GTG GCC TTC ATG AAA GGG CGC GGC ATC CCG    385
 Y   N   V   V   A   V   A   F   M   K   G   R   G   I   P     75

ACC TTC CAG CCA TAC AAC CTG TCC GAC GCG GAG TTT CGC CGC CAG    430
 T   F   Q   P   Y   N   L   S   D   A   E   F   R   R   Q     90

GTC GGC GTG CTC AAC GCC CAG GGC CGC GCG GTG CTG ATT TCT CTG    475
 V   G   V   L   N   A   Q   G   R   A   V   L   I   S   L    105

GGG GGC GCC GAC GCG CAC ATC GAG TTG CAC GCC GGG CAG GAG CAG    520
 G   G   A   D   A   H   I   E   L   H   A   G   Q   E   Q    120

GCG CTG GCC GCC GAG ATC GTC CGC CTG GTG GAA ACC TAC GGT TTC    565
 A   L   A   A   E   I   V   R   L   V   E   T   Y   G   F    135

GAC GGC CTG GAC ATC GAC CTC GAG CAG AGC GCC ATC GAC CTG GCC    610
 D   G   L   D   I   D   L   E   Q   S   A   I   D   L   A    150

GAC AAC CAG CGG GTG CTG CCG GCG GCC CTC AAG CTG GTG CGC GAG    655
 D   N   Q   R   V   L   P   A   A   L   K   L   V   R   E    165

CAC TAC GCC GGG CAG GGC AAG CAC TTC ATC GTC AGC ATG GCC CCG    700
 H   Y   A   G   Q   G   K   H   F   I   V   S   M   A   P    180

GAG TTT CCC TAT CTG CAC AAG AAC GGC AAG TAC GTG CCT TAT CTG    745
 E   F   P   Y   L   H   K   N   G   K   Y   V   P   Y   L    195
```

Figure 14 continued

```
CAG GCC CTG GAA GGC GTC TAC GAC TTC ATC GCG CCG CAG TAC TAC    790
 Q   A   L   E   G   V   Y   D   F   I   A   P   Q   Y   Y    210

AAC CAG GGC GGC GAC GGC CTG TGG GTC CAG GAG GCG AAC GGC GGC    835
 N   Q   G   G   D   G   L   W   V   Q   E   A   N   G   G    225

AAG GGC GCC TGG ATC GCG CAG AAC AAC GAC GCG ATG AAA GAA GAC    880
 K   G   A   W   I   A   Q   N   N   D   A   M   K   E   D    240

TTC CTC TAC TAC CTC ACC GAG AGC CTG GCC ACC GGC AGC CGC GAC    925
 F   L   Y   Y   L   T   E   S   L   A   T   G   S   R   D    255

TTC GTG CGG ATC CCG GCG CAG CGC CTG GCC ATC GGC CTG CCG AGC    970
 F   V   R   I   P   A   Q   R   L   A   I   G   L   P   S    270

AAC GTC GAT GCG GCG GCT ACC GGC TAC GTG ATC GAT CCC GCC GCG   1015
 N   V   D   A   A   A   T   G   Y   V   I   D   P   A   A    285

GTG AGC AAC GCG TTT CGC CGC CTG GAA GCC GCC GGT CAC GCG ATC   1060
 V   S   N   A   F   R   R   L   E   A   A   G   H   A   I    300

AAG GGC CTG ATG ACC TGG TCG GTG AAC TGG GAC GAT GGC CTG AAC   1105
 K   G   L   M   T   W   S   V   N   W   D   D   G   L   N    315

AAG CGC GGC GAG CGC TAC AAC TGG GAG TTC CGC AAG CGC TAC GCC   1150
 K   R   G   E   R   Y   N   W   E   F   R   K   R   Y   A    330

AGC CTC ATC CAT GAC GGC GAG GGC GGC GAC CAG CGC CCG GCG GCG   1195
 S   L   I   H   D   G   E   G   G   D   Q   R   P   A   A    345

CCG CAG GGC CTG CGT CTG CTG GAG CGC GGC GAG ACC AGC CTG GTG   1240
 P   Q   G   L   R   L   L   E   R   G   E   T   S   L   V    360

CTG GCC TGG AAC GCC TCC AGC GGG CAG CGT CCG ATC GAT TAC TAC   1285
 L   A   W   N   A   S   S   G   Q   R   P   I   D   Y   Y    375

AGC CTC TAT CGC GAC GGC GCC ATG GTT GGC CAG AGC GCC GCG CTG   1330
 S   L   Y   R   D   G   A   M   V   G   Q   S   A   A   L    390

GGT TCC ACC GAC AGC GGC CTG ACG GCG GAC ACC CGC TAC AGT TAT   1375
 G   S   T   D   S   G   L   T   A   D   T   R   Y   S   Y    405
```

Figure 14 continued

```
TTC GTC ACC GCC ACC GAT ACC CAG GGC AAC CAG TCG CTG CCC AGC   1420
F   V   T   A   T   D   T   Q   G   N   Q   S   L   P   S     420

GAA GGC CTG GAG GTC AGC ACC AGC GGC GGG GCG GTG GAT CCG CAA   1465
 E   G   L   E   V   S   T   S   G   G   A   V   D   P   Q     435

TTC CCG CAA TGG CGG GAG AAC CAG GCC TAT CGG GTC GAC GAT GGG   1510
 F   P   Q   W   R   E   N   Q   A   Y   R   V   D   D   G     450

GTG ACC TAC GAG GGG CTG CGC TAT CTC TGC CTG CAG GCG CAC ACC   1555
 V   T   Y   E   G   L   R   Y   L   C   L   Q   A   H   T     465

TCA AAC AGC GGC TGG ACG CCG CCG GTA GCC TTC ACC CTC TGG CGG   1600
 S   N   S   G   W   T   P   P   V   A   F   T   L   W   R     480

CCG CTG CGC TGA                                              1645
 P   L   R   *                                                 484

GGGGGCGGGCCGGTCCGCGCTGCGGGCCGGTCCTTTGGCCGGCGAGAGCCACGCACGGGTGGCGCCATATCCATATG
CGTTTTCGGTATTTCCGCTTGATCGTTCATGGCGTTTACCATCACGGTATAACTTGTTATAACAAGCATTCGAGCCA
CTCCTGCAAGCGAATCCGACCATGGGCAAACTGCTCCCGCTTTCCCCCGTGCCGCTGTACAGCCAGCTCAAGGAGCT
GCTGCGCGGACGCAT                                                              1858
```

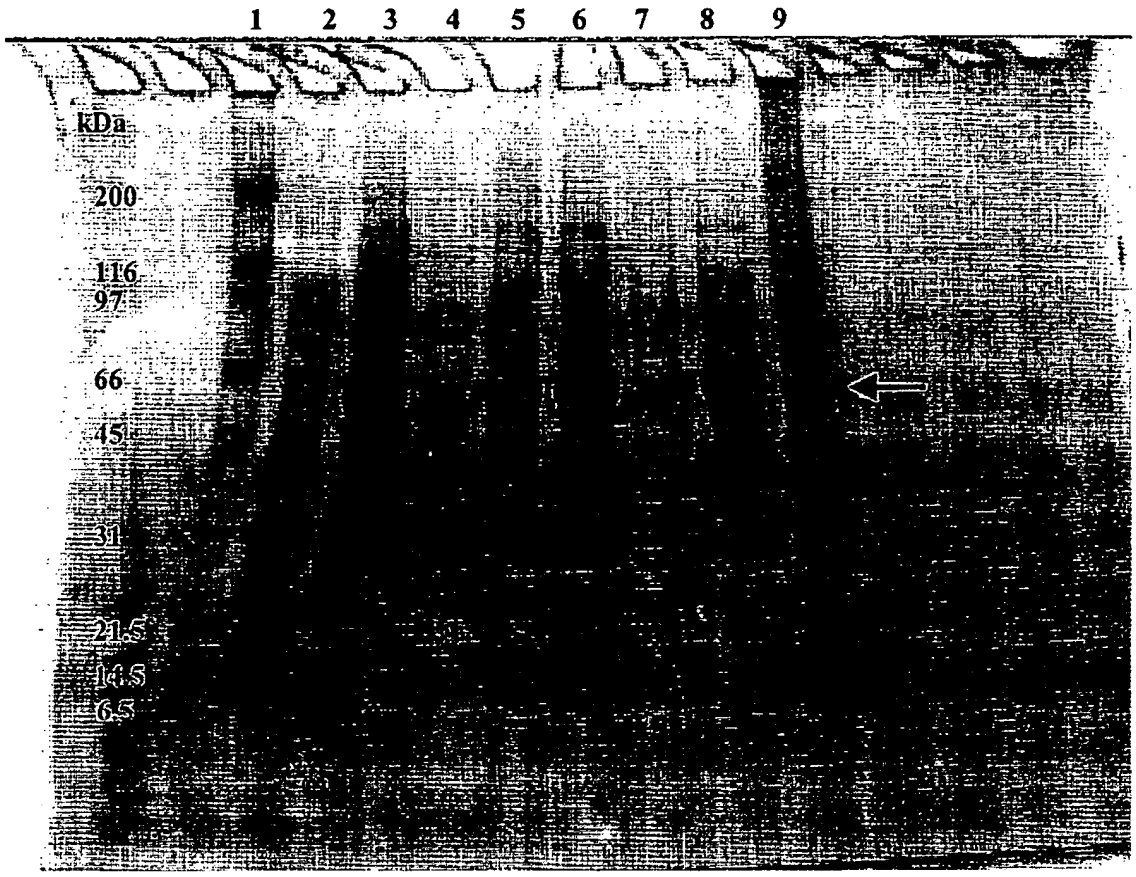

Figure 15

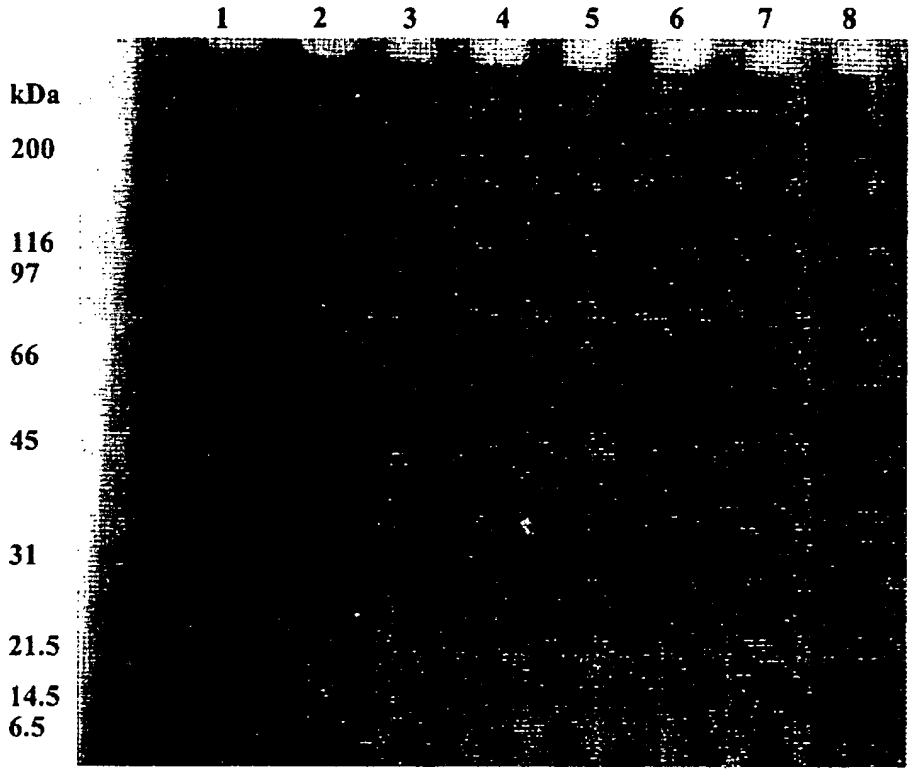

Figure 16

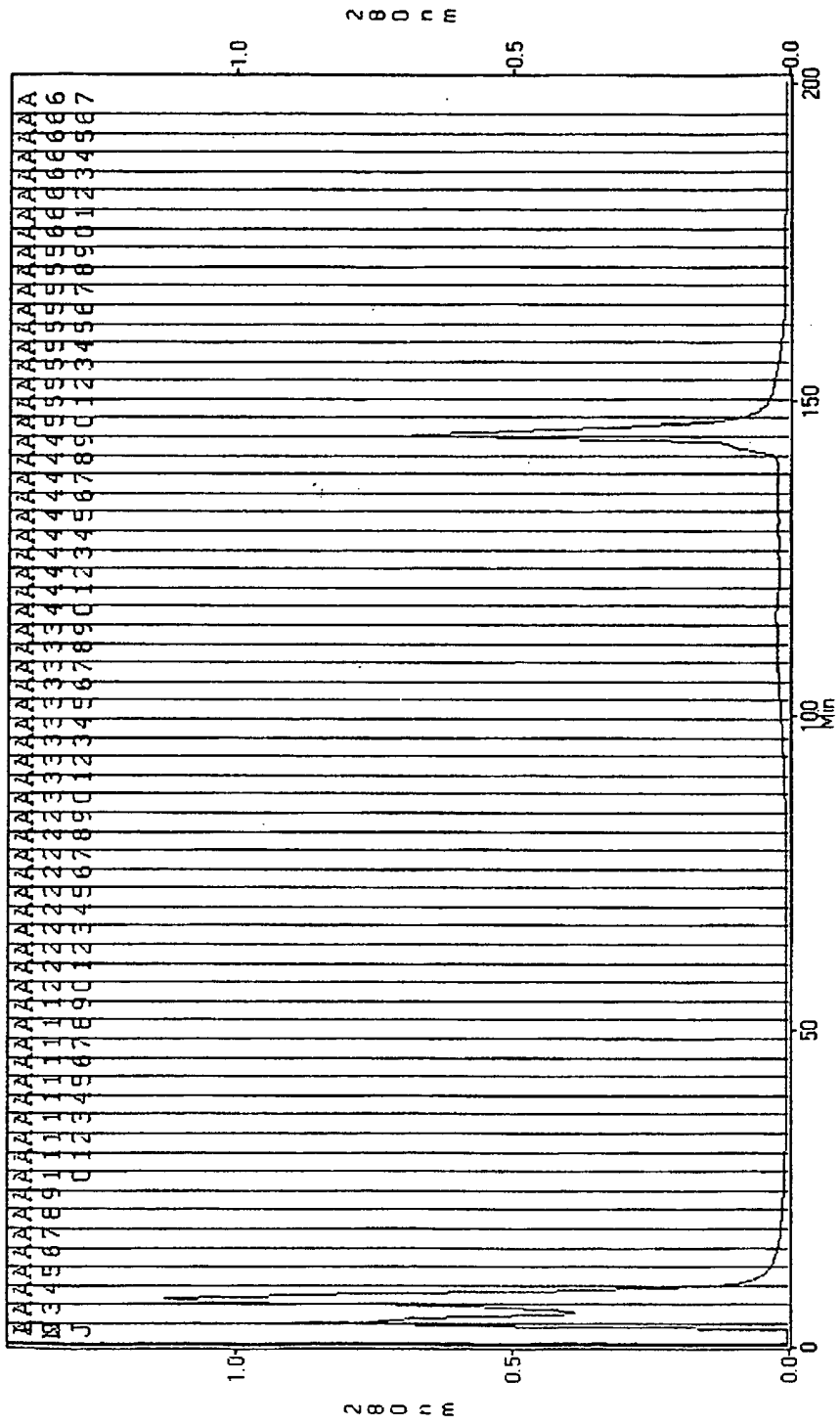

Figure 17

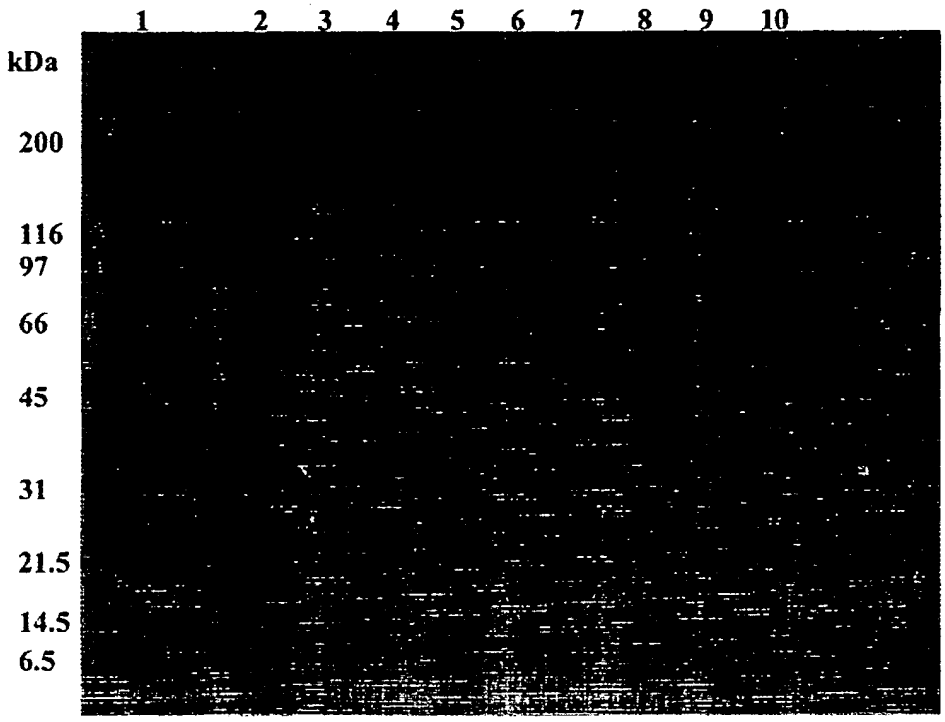

Figure 18

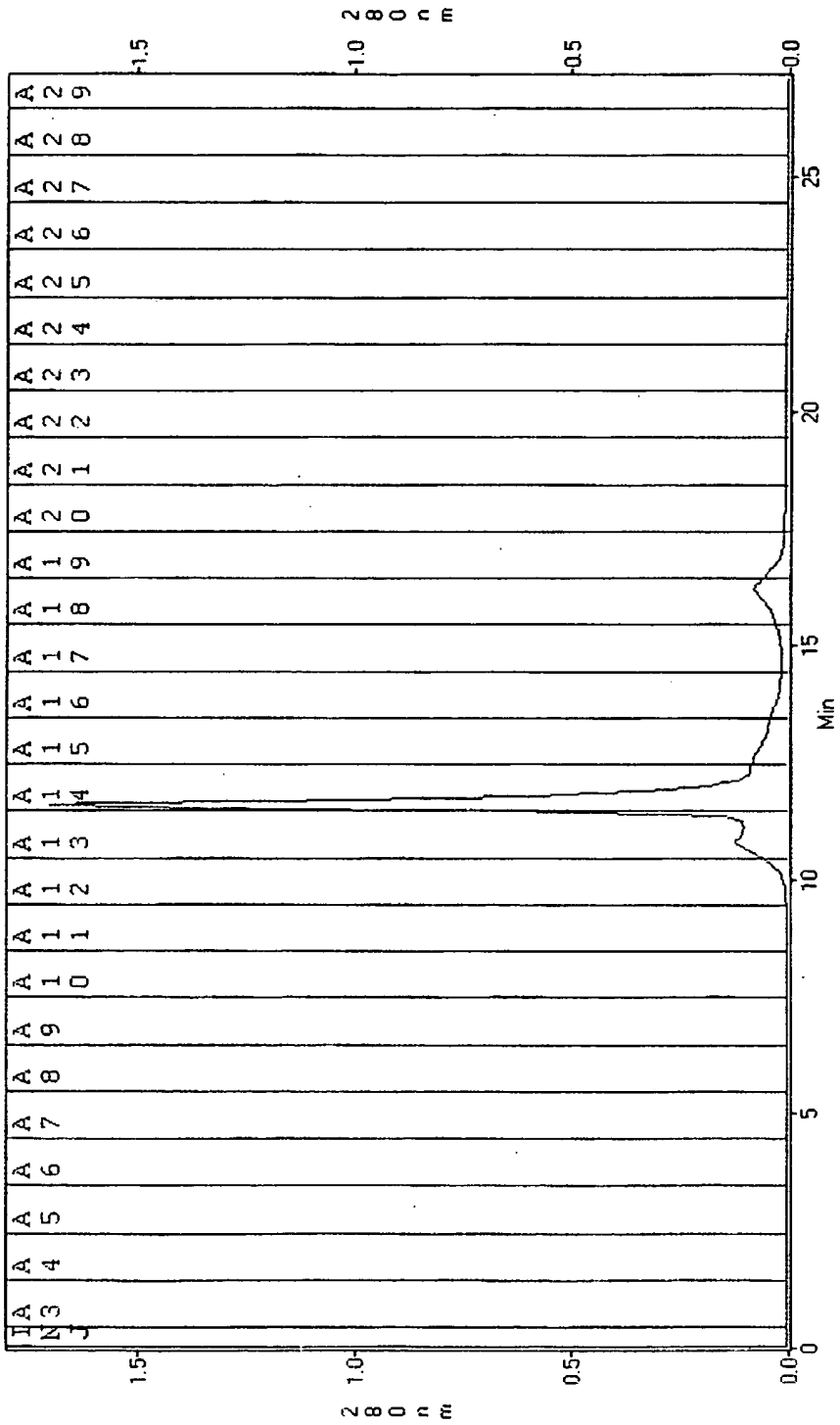

Figure 19

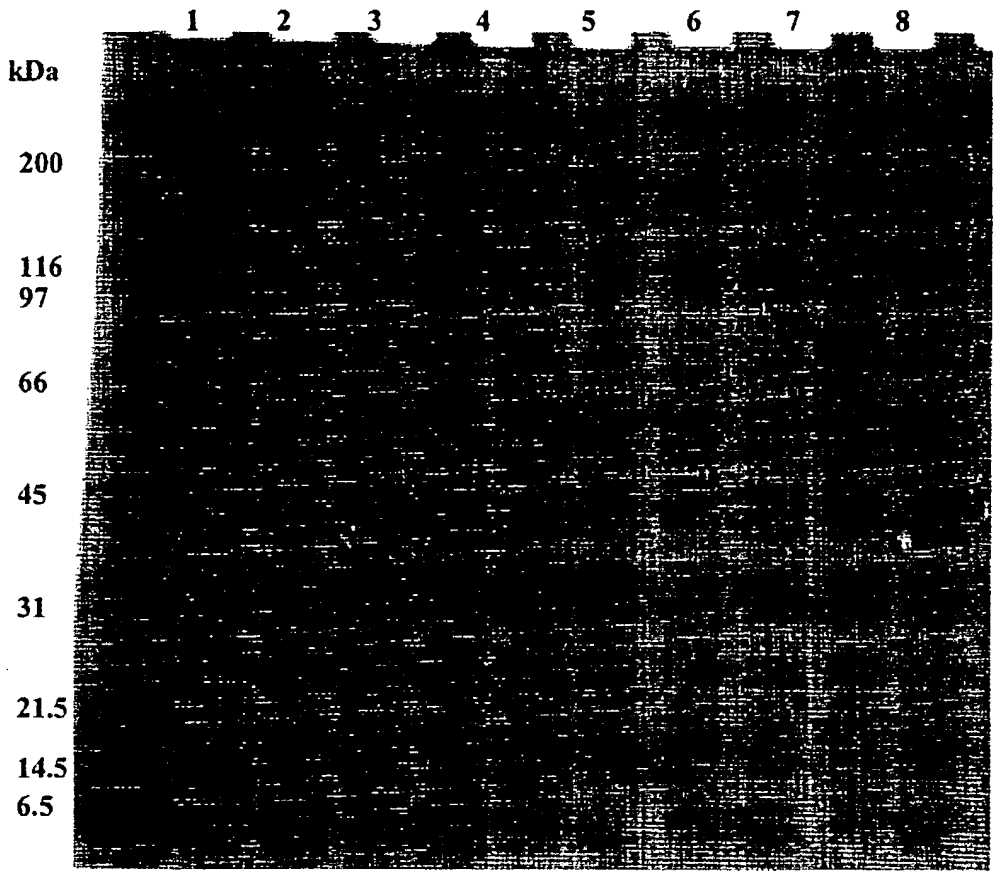

Figure 20

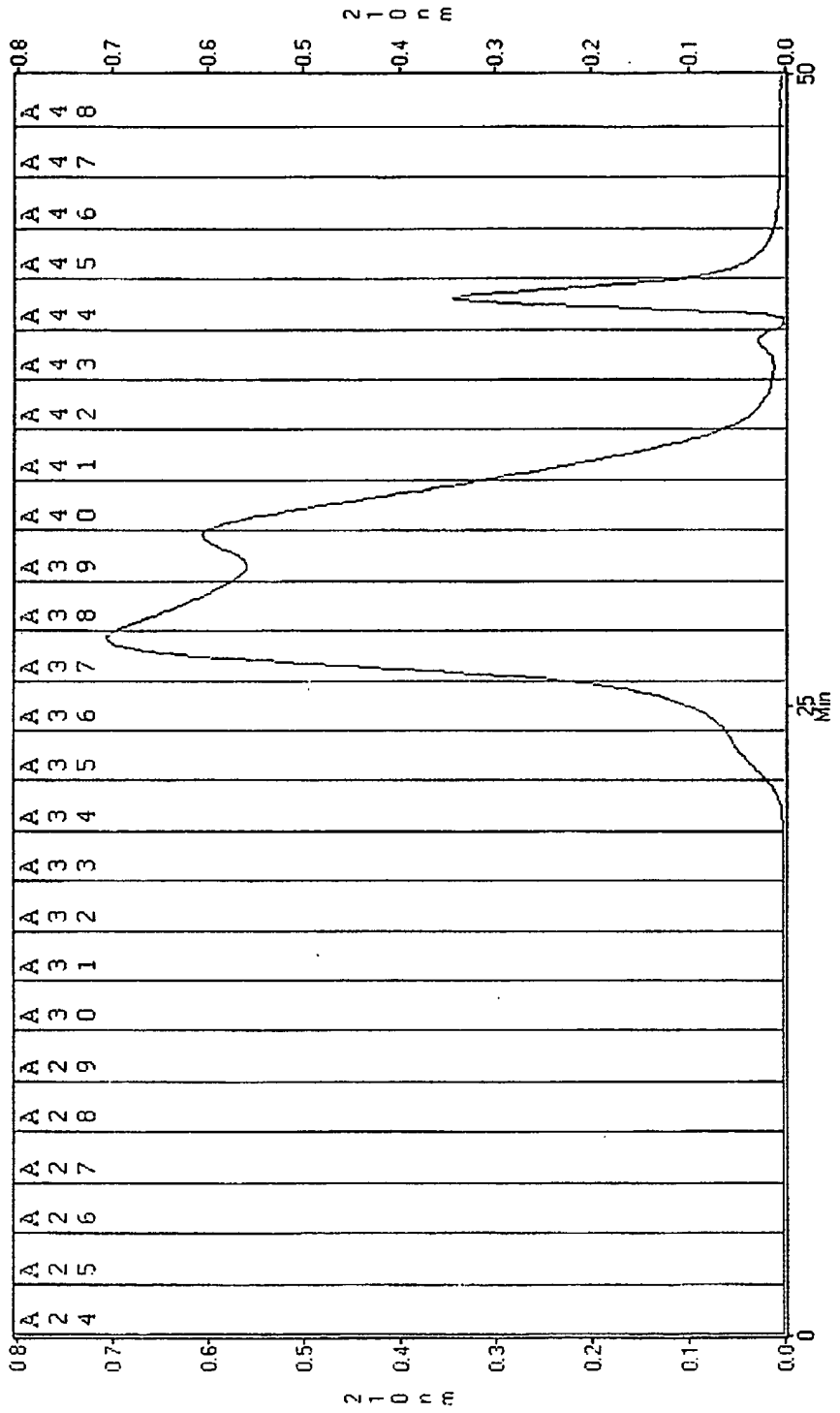
210nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
50
0
25
Min
A 2 4
A 2 5
A 2 6
A 2 7
A 2 8
A 2 9
A 3 0
A 3 1
A 3 2
A 3 3
A 3 4
A 3 5
A 3 6
A 3 7
A 3 8
A 3 9
A 4 0
A 4 1
A 4 2
A 4 3
A 4 4
A 4 5
A 4 6
A 4 7
A 4 8

Figure 21

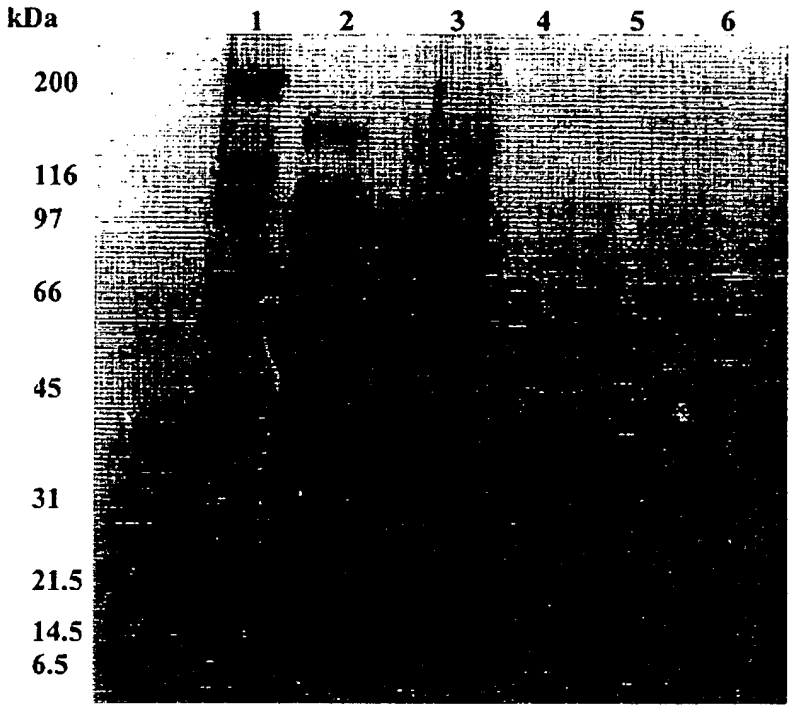

Figure 22

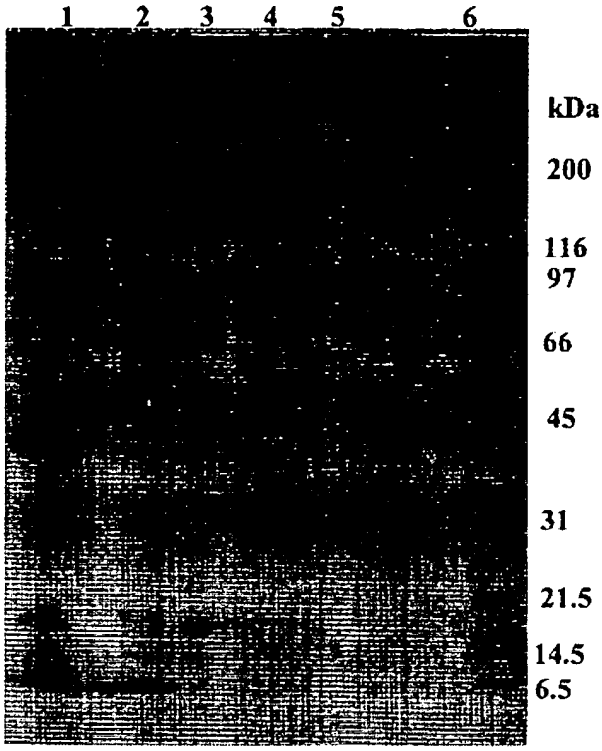

Figure 23

Figure 24

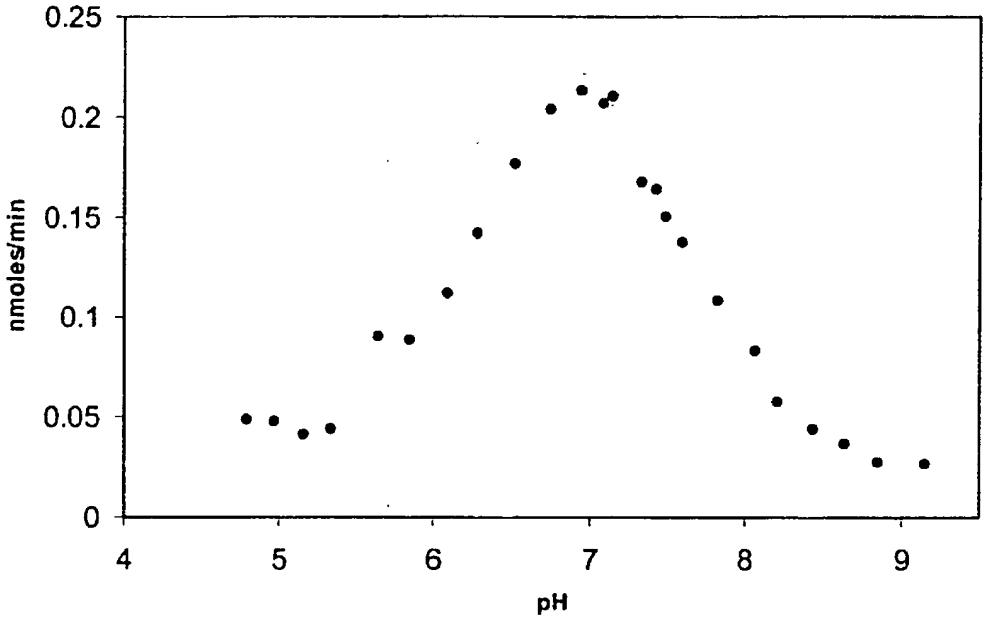

Figure 25

```
AACTGCTGGTGATGGGCGAGTCCGAAATCCTCGCCGTCCTGGAAGACTGATCGGTCTCACCACTCCGTTTTCACCGA
ATTCGATTTAGAGGAAAGAGAAC                                                  100

ATG GCT GCC AAA GAA GTT AAG TTC GGC GAT TCC GCT CGC AAG AAA    145
 M   A   A   K   E   V   K   F   G   D   S   A   R   K   K     15

ATG CTG GTC GGC GTG AAC GTG CTG GCC GAT GCC GTC AAG GCC ACC    190
 M   L   V   G   V   N   V   L   A   D   A   V   K   A   T     30

CTC GGC CCG AAA GGC CGC AAC GTG GTT CTG GAC AAG TCC TTC GGC    235
 L   G   P   K   G   R   N   V   V   L   D   K   S   F   G     45

GCT CCG ACC ATC ACC AAG GAC GGC GTT TCC GTC GCC AAG GAA ATC    280
 A   P   T   I   T   K   D   G   V   S   V   A   K   E   I     60

GAG CTG AAA GAC AAG TTC GAG AAC ATG GGC GCG CAA CTG GTG AAA    325
 E   L   K   D   K   F   E   N   M   G   A   Q   L   V   K     75

GAC GTT GCC TCC AAG GCC AAC GAC GCT GCC GGT GAC GGC ACC ACC    370
 D   V   A   S   K   A   N   D   A   A   G   D   G   T   T     90

ACC GCG ACC GTC CTG GCC CAG GCC ATC GTC AAC GAA GGC CTG AAG    415
 T   A   T   V   L   A   Q   A   I   V   N   E   G   L   K    105

GCC GTT GCC GCC GGC ATG AAC CCG ATG GAC CTG AAG CGT GGC ATC    460
 A   V   A   A   G   M   N   P   M   D   L   K   R   G   I    120

GAC AAG GCC ACC GTG GCC ATC GTC GCC CAG CTG AAA GAG CTG GCC    505
 D   K   A   T   V   A   I   V   A   Q   L   K   E   L   A    135

AAG CCC TGC GCC GAC ACC AAG GCC ATC GCC CAG GTA GGC ACC ATC    550
 K   P   C   A   D   T   K   A   I   A   Q   V   G   T   I    150

TCC GCC AAC TCC GAC GAG TCC ATC GGC CAG ATC ATT GCC GAA GCC    595
 S   A   N   S   D   E   S   I   G   Q   I   I   A   E   A    165

ATG GAA AAA GTC GGT AAA GAA GGC GTG ATC ACC GTC GAG GAG GGC    640
 M   E   K   V   G   K   E   G   V   I   T   V   E   E   G    180

TCG GGC CTG GAA AAC GAA CTG TCC GTC GTC GAA GGC ATG CAG TTC    685
 S   G   L   E   N   E   L   S   V   V   E   G   M   Q   F    195
```

Figure 25 continued

```
GAT CGC GGC TAC CTG TCC CCC TAC TTC GTG AAC AAG CCG GAC ACC    730
 D   R   G   Y   L   S   P   Y   F   V   N   K   P   D   T    210

ATG GCT GCC GAG CTG GAT AGC CCG CTG CTG CTG CTG GTC GAC AAG    775
 M   A   A   E   L   D   S   P   L   L   L   L   V   D   K    225

AAG ATC TCC AAC ATC CGC GAA ATG CTG CCG GTG CTG GAA GCC GTC    820
 K   I   S   N   I   R   E   M   L   P   V   L   E   A   V    240

GCC AAG GCC GGC CGT CCG CTG CTG ATC GTC GCC GAG GAC GTC GAG    865
 A   K   A   G   R   P   L   L   I   V   A   E   D   V   E    255

GGC GAA GCC CTG GCC ACC CTG GTG GTG AAC AAC ATG CGT GGC ATC    910
 G   E   A   L   A   T   L   V   V   N   N   M   R   G   I    270

GTC AAG GTC GCG GCT GTC AAG GCT CCG GGC TTC GGC GAT CGC CGC    955
 V   K   V   A   A   V   K   A   P   G   F   G   D   R   R    285

AAG GCC ATG CTG CAG GAC ATC GCC ATC CTC ACC GGC GGT ACC GTG   1000
 K   A   M   L   Q   D   I   A   I   L   T   G   G   T   V    300

ATC AGC GAA GAA GTC GGC CTG AGC CTG GAA GGC GCT ACC CTG GAG   1045
 I   S   E   E   V   G   L   S   L   E   G   A   T   L   E    315

CAC CTG GGC AAC GCC AAG CGC GTC GTG ATC AAC AAG GAA AAC ACC   1090
 H   L   G   N   A   K   R   V   V   I   N   K   E   N   T    330

ACC ATC ATC GAT GGC GCC GGT GTG CAG GCT GAT ATC GAA GCC CGC   1135
 T   I   I   D   G   A   G   V   Q   A   D   I   E   A   R    345

GTC CTG CAG ATC CGC AAG CAG ATC GAG GAA ACC ACT TCC GAC TAC   1180
 V   L   Q   I   R   K   Q   I   E   E   T   T   S   D   Y    360

GAC CGC GAG AAG CTG CAA GAG CGC CTG GCC AAG CTG GCC GGC GGT   1225
 D   R   E   K   L   Q   E   R   L   A   K   L   A   G   G    375

GTT GCC GTG ATC AAG GTA GGC GCT GCC ACC GAA GTC GAG ATG AAA   1270
 V   A   V   I   K   V   G   A   A   T   E   V   E   M   K    390

GAG AAG AAA GCC CGC GTC GAA GAC GCC CTG CAC GCT ACC CGT GCA   1315
 E   K   K   A   R   V   E   D   A   L   H   A   T   R   A    405
```

Figure 25 continued

```
GCG GTG GAA GAG GGC GTG GTT CCC GGC GGC GGC GTA GCC CTG GTT    1360
 A   V   E   E   G   V   V   P   G   G   G   V   A   L   V      420

CGT GCC CTG CAA GCC ATC GAA GGC CTG AAG GGT GAC AAC GAG GAG    1405
 R   A   L   Q   A   I   E   G   L   K   G   D   N   E   E      435

CAG AAC GTC GGT ATC GCC CTG CTG CGT CGC GCC GTC GAA TCG CCG    1450
Q   N   V   G   I   A   L   L   R   R   A   V   E   S   P      450

CTG CGC CAG ATC GTG GCC AAC GCC GGC GAC GAG CCG AGC GTA GTG    1495
 L   R   Q   I   V   A   N   A   G   D   E   P   S   V   V      465

GTC GAC AAG GTC AAG CAG GGT TCC GGC AAC TAC GGC TTC AAC GCT    1540
 V   D   K   V   K   Q   G   S   G   N   Y   G   F   N   A      480

GCT ACC GGC GTG TAC GGC GAC ATG ATC GAG ATG GGC ATC CTG GAC    1585
 A   T   G   V   Y   G   D   M   I   E   M   G   I   L   D      495

CCG GCC AAG GTC ACT CGT TCC GCT CTG CAG GCT GCG GCC TCC ATC    1630
 P   A   K   V   T   R   S   A   L   Q   A   A   A   S   I      510

GGC GGT CTG ATG ATC ACC ACC GAA GCC ATG GTT GCC GAG ATC GTG    1675
G   G   L   M   I   T   T   E   A   M   V   A   E   I   V      525

GAA GAC AAG CCT GCC ATG GGC GGC ATG CCT GAC ATG GGC GGC ATG    1720
 E   D   K   P   A   M   G   G   M   P   D   M   G   G   M      540

GGC GGC ATG GGC GGC ATG ATG TAA                               1744
 G   G   M   G   G   M   M   *                                  548

GCCGACCGGCCCCTGTGGTTGGAAAAGCCCCGCTTCGGCGGGGCTTTTTTATTCCTCAGTGCGGGCAGTCCTGGCGG
TAGCCCGGCAGCTCCCTTGCCGCCGG          1847
```

Figure 26

```
ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG      45
 M   G   G   S   H   H   H   H   H   H   G   M   A   S   M      15

ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT      90
 T   G   G   Q   Q   M   G   R   D   L   Y   D   D   D   D      30

ATG GCT GCC AAA GAA GTT AAG TTC GGC GAT TCC GCT CGC AAG AAA     135
 M   A   A   K   E   V   K   F   G   D   S   A   R   K   K      45

ATG CTG GTC GGC GTG AAC GTG CTG GCC GAT GCC GTC AAG GCC ACC     180
 M   L   V   G   V   N   V   L   A   D   A   V   K   A   T      60

CTC GGC CCG AAA GGC CGC AAC GTG GTT CTG GAC AAG TCC TTC GGC     225
 L   G   P   K   G   R   N   V   V   L   D   K   S   F   G      75

GCT CCG ACC ATC ACC AAG GAC GGC GTT TCC GTC GCC AAG GAA ATC     270
 A   P   T   I   T   K   D   G   V   S   V   A   K   E   I      90

GAG CTG AAA GAC AAG TTC GAG AAC ATG GGC GCG CAA CTG GTG AAA     315
 E   L   K   D   K   F   E   N   M   G   A   Q   L   V   K     105

GAC GTT GCC TCC AAG GCC AAC GAC GCT GCC GGT GAC GGC ACC ACC     330
 D   V   A   S   K   A   N   D   A   A   G   D   G   T   T     120

ACC GCG ACC GTC CTG GCC CAG GCC ATC GTC AAC GAA GGC CTG AAG     375
 T   A   T   V   L   A   Q   A   I   V   N   E   G   L   K     135

GCC GTT GCC GCC GGC ATG AAC CCG ATG GAC CTG AAG CGT GGC ATC     450
A   V   A   A   G   M   N   P   M   D   L   K   R   G   I     150

GAC AAG GCC ACC GTG GCC ATC GTC GCC CAG CTG AAA GAG CTG GCC     495
 D   K   A   T   V   A   I   V   A   Q   L   K   E   L   A     165

AAG CCC TGC GCC GAC ACC AAG GCC ATC GCC CAG GTA GGC ACC ATC     540
 K   P   C   A   D   T   K   A   I   A   Q   V   G   T   I     180

TCC GCC AAC TCC GAC GAG TCC ATC GGC CAG ATC ATT GCC GAA GCC     585
 S   A   N   S   D   E   S   I   G   Q   I   I   A   E   A     195

ATG GAA AAA GTC GGT AAA GAA GGC GTG ATC ACC GTC GAG GAG GGC     630
 M   E   K   V   G   K   E   G   V   I   T   V   E   E   G     210

TCG GGC CTG GAA AAC GAA CTG TCC GTC GTC GAA GGC ATG CAG TTC     675
 S   G   L   E   N   E   L   S   V   V   E   G   M   Q   F     225
```

Figure 26 continued

```
GAT CGC GGC TAC CTG TCC CCC TAC TTC GTG AAC AAG CCG GAC ACC   720
 D   R   G   Y   L   S   P   Y   F   V   N   K   P   D   T   240

ATG GCT GCC GAG CTG GAT AGC CCG CTG CTG CTG CTG GTC GAC AAG   765
 M   A   A   E   L   D   S   P   L   L   L   L   V   D   K   255

AAG ATC TCC AAC ATC CGC GAA ATG CTG CCG GTG CTG GAA GCC GTC   810
 K   I   S   N   I   R   E   M   L   P   V   L   E   A   V   270

GCC AAG GCC GGC CGT CCG CTG CTG ATC GTC GCC GAG GAC GTC GAG   855
 A   K   A   G   R   P   L   L   I   V   A   E   D   V   E   385

GGC GAA GCC CTG GCC ACC CTG GTG GTG AAC AAC ATG CGT GGC ATC   900
 G   E   A   L   A   T   L   V   V   N   N   M   R   G   I   300

GTC AAG GTC GCG GCT GTC AAG GCT CCG GGC TTC GGC GAT CGC CGC   945
 V   K   V   A   A   V   K   A   P   G   F   G   D   R   R   315

AAG GCC ATG CTG CAG GAC ATC GCC ATC CTC ACC GGC GGT ACC GTG   990
 K   A   M   L   Q   D   I   A   I   L   T   G   G   T   V   330

ATC AGC GAA GAA GTC GGC CTG AGC CTG GAA GGC GCT ACC CTG GAG  1035
 I   S   E   E   V   G   L   S   L   E   G   A   T   L   E   345

CAC CTG GGC AAC GCC AAG CGC GTC GTG ATC AAC AAG GAA AAC ACC  1080
 H   L   G   N   A   K   R   V   V   I   N   K   E   N   T   360

ACC ATC ATC GAT GGC GCC GGT GTG CAG GCT GAT ATC GAA GCC CGC  1125
 T   I   I   D   G   A   G   V   Q   A   D   I   E   A   R   375

GTC CTG CAG ATC CGC AAG CAG ATC GAG GAA ACC ACT TCC GAC TAC  1170
 V   L   Q   I   R   K   Q   I   E   E   T   T   S   D   Y   390

GAC CGC GAG AAG CTG CAA GAG CGC CTG GCC AAG CTG GCC GGC GGT  1215
 D   R   E   K   L   Q   E   R   L   A   K   L   A   G   G   405

GTT GCC GTG ATC AAG GTA GGC GCT GCC ACC GAA GTC GAG ATG AAA  1260
 V   A   V   I   K   V   G   A   A   T   E   V   E   M   K   420

GAG AAG AAA GCC CGC GTC GAA GAC GCC CTG CAC GCT ACC CGT GCA  1305
 E   K   K   A   R   V   E   D   A   L   H   A   T   R   A   435
```

Figure 26 continued

```
GCG GTG GAA GAG GGC GTG GTT CCC GGC GGC GGC GTA GCC CTG GTT   1350
 A   V   E   E   G   V   V   P   G   G   G   V   A   L   V     450

CGT GCC CTG CAA GCC ATC GAA GGC CTG AAG GGT GAC AAC GAG GAG   1395
 R   A   L   Q   A   I   E   G   L   K   G   D   N   E   E     465

CAG AAC GTC GGT ATC GCC CTG CTG CGT CGC GCC GTC GAA TCG CCG   1440
 Q   N   V   G   I   A   L   L   R   R   A   V   E   S   P     480

CTG CGC CAG ATC GTG GCC AAC GCC GGC GAC GAG CCG AGC GTA GTG   1485
 L   R   Q   I   V   A   N   A   G   D   E   P   S   V   V     495

GTC GAC AAG GTC AAG CAG GGT TCC GGC AAC TAC GGC TTC AAC GCT   1530
 V   D   K   V   K   Q   G   S   G   N   Y   G   F   N   A     510

GCT ACC GGC GTG TAC GGC GAC ATG ATC GAG ATG GGC ATC CTG GAC   1575
 A   T   G   V   Y   G   D   M   I   E   M   G   I   L   D     525

CCG GCC AAG GTC ACT CGT TCC GCT CTG CAG GCT GCG GCC TCC ATC   1620
 P   A   K   V   T   R   S   A   L   Q   A   A   A   S   I     540

GGC GGT CTG ATG ATC ACC ACC GAA GCC ATG GTT GCC GAG ATC GTG   1665
 G   G   L   M   I   T   T   E   A   M   V   A   E   I   V     555

GAA GAC AAG CCT GCC ATG GGC GGC ATG CCT GAC ATG GGC GGC ATG   1710
 E   D   K   P   A   M   G   G   M   P   D   M   G   G   M     570

GGC GGC ATG ATG TAA                                          1725
 G   G   M   M   *                                            575
```

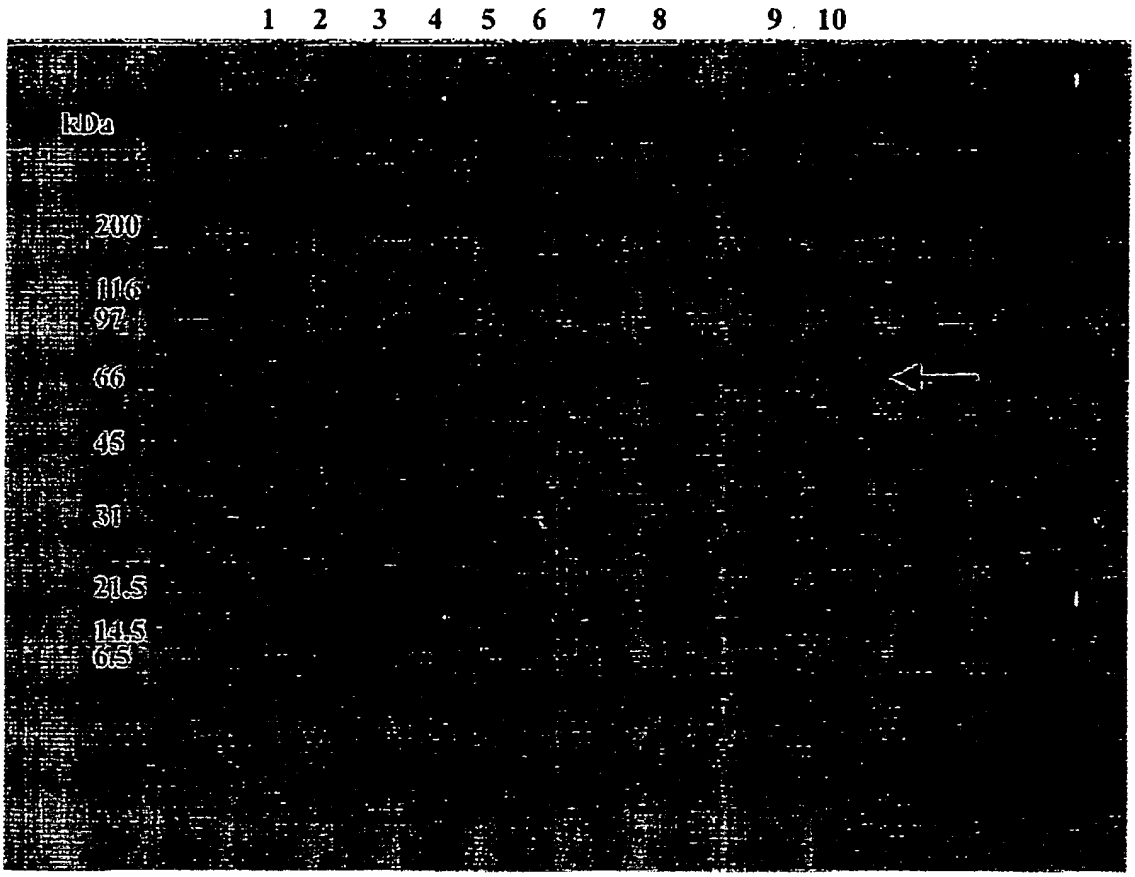

Figure 27

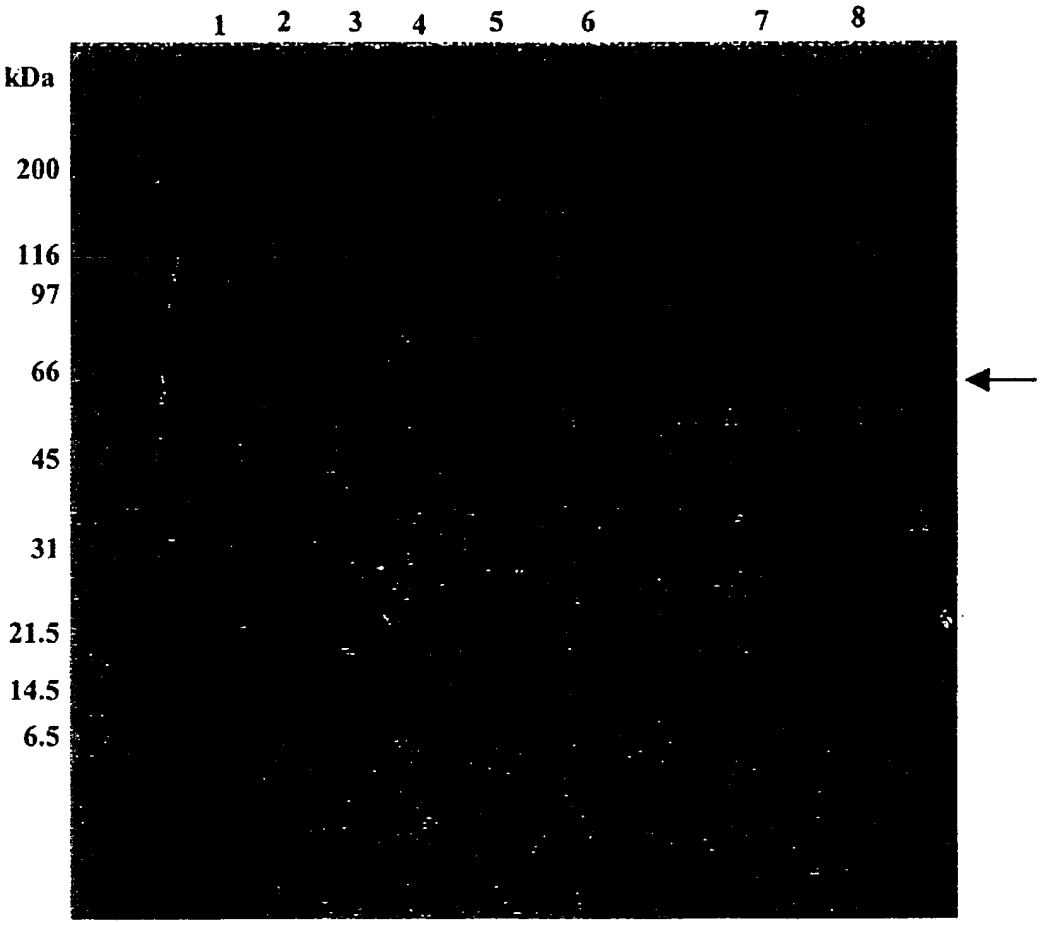

Figure 28

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/02554

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/31 C12N15/56 C07K14/21 C12N9/24 A61K39/104
A61K38/47 A61K39/395 G01N33/569 C07K16/12 C07K16/40
C12N15/70 C12N1/21 A61K48/00 C12Q1/68 C12Q1/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EMBL, EPO-Internal, WPI Data, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ATTILA SIPOS ET AL.: "Cloning and sequencing of the genes coding for the 10- and 60-kDa heat shock proteins from Pseudomonas aeruginosa and mapping of a species-specific epitope" INFECTION AND IMMUNITY, vol. 59, no. 9, September 1991 (1991-09), pages 3219-3226, XP002147873 page 3221, left-hand column, last paragraph -page 3225, right-hand column, last paragraph; figure 2 --- -/--	1,3-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
14 November 2000	01. 12. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Montero Lopez, B

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/02554

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PHILIPP HÜBNER ET AL.: "Molecular analysis of the Rhodobacter capsulatus chaperonin (groESL) operon: purification and characterization of Cpn60" ARCHIVES OF MICROBIOLOGY, vol. 166, 1996, pages 193-203, XP000938358 page 195, right-hand column, last paragraph -page 196, left-hand column, paragraph 3 page 197, left-hand column, paragraph 1 -right-hand column, paragraph 2; figure 4 ---	1,3-8, 13,26-28
X	EP 0 543 091 A (RIEDEL-DE HAEN AKTIENGESELLSCHAFT) 26 May 1993 (1993-05-26) page 2, line 14 - line 23 page 3, line 14 - line 22; examples 2,3 ---	4-6,8-31
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 November 2000	

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EDELTRAUD LÜNEBERG ET AL.: "Monoclonal antibody against species-specific epitope of Pseudomonas aeruginosa Hsp60 protein cross-reacts with Pseudomonas stutzeri and other Pseudomonas species" FEMS MICROBIOLOGY LETTERS, vol. 154, 1997, pages 131-137, XP000939293 figure 3 ---	1,3-31
X	DATABASE EMBL 'Online! Accession number P77829, 1 November 1997 (1997-11-01) XP002147874 the whole document -& FRANZ NARBERHAUS ET AL.: "The Bradyrhizobium japonicumrphH1 gene encoding a gamma32-like protein is a part of a unique heat shock gene cluster together with groESL1 and three small heat shock genes" JOURNAL OF BACTERIOLOGY, vol. 178, no. 18, September 1996 (1996-09), pages 5337-5346, XP002147987 ---	1,4-8, 13,19-22
X	DATABASE WPI Section Ch, Week 199820 Derwent Publications Ltd., London, GB; Class B04, AN 1998-224333 XP002152721 WANG S L: "Chitinase preparation" & JP 10 066568 A (WANG S L), 10 March 1998 (1998-03-10) abstract ---	1,2,4,5, 31
X	DATABASE SWALL 'Online! Database Entry 027522, 1 January 1998 (1998-01-01) SMITH D.R. ET AL.: "Conserved protein" XP002152720 the whole document ---	1,2,4
X	SANG WAN GAL ET AL.: "Cloning of the 52-kDa chitinase gene from Serratia marcescens KCTC2172 and its proteolytic cleavage into an active 35kDa enzyme" FEMS MICROBIOLOGY LETTERS, vol. 160, 1998, pages 151-158, XP000960609 abstract page 153, right-hand column, paragraph 2 -page 156, left-hand column, paragraph 1; figure 1 --- -/--	4,5, 26-28

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 114 712 A (TAMOTSU FUKUDA ET AL.) 19 May 1992 (1992-05-19) column 2, line 42 -column 3, line 18 column 6, line 17 -column 14, line 10	1,3-31
A	WO 98 32769 A (AUSPHARM INTERNATIONAL LIMITED) 30 July 1998 (1998-07-30) cited in the application page 2, line 1 -page 4, line 35; examples	1,3-31
A	PER JENSEN ET AL.: "Cloning and nucleotide sequence comparison of the groE operon of Pseudomonas aeruginosa and Burkholderia cepacia" APMIS, vol. 103, no. 2, February 1995 (1995-02), pages 113-123, XP000938353 page 113, right-hand column, last paragraph -page 114, left-hand column, paragraph 3; figures 2,3 page 118, left-hand column, paragraph 2 -page 122, right-hand column, paragraph 1	1,3-31
A	CHANPEN WIWAT ET AL.: "Expression of chitinase-encoding genes from Aeromonas hydrophila and Pseudomonas maltophilia in Bacillus thurigiensis subsp. israelensis" GENE, vol. 179, 1996, pages 119-126, XP004071973 AMSTERDAM NL abstract page 121, right-hand column, paragraph 1 -page 125, left-hand column, paragraph 1	1,2,4,5, 26-28,30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 00/02554

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 22-25, and claim 18, as far as concerning an in vivo method, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compounds/compositions.

2. [X] Claims Nos.: 32
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 32

Present claim 32 relates to an agent defined by reference to a desirable characteristic or property, namely being capable of antagonising, inhibiting or otherwise interfering with the function or expression of a protein as defined in claims 1-5. The claim covers all agents having this characteristic or property, whereas the application does not provide support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for any specific example of such agents. In the present case, the claim so lacks support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claim also lacks clarity (Article 6 PCT). An attempt is made to define the agent by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, no search has been carried out for claim 32.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 3 and partially 1, 4-31

P. aeruginosa HSP60 protein having N-terminal sequence I, fragments and antibodies thereof; antigenic composition comprising the same; uses thereof for the detection, diagnostic, prophylaxis and treatmentof P. aeruginosa; nucleic acid molecule encoding the protein, derivatives and analogs thereof; vector and host cell comprising the same and use thereof in a vaccine composition and for the detection/diagnosis of P. aeruginosa; test for determining if the protein or fragments represent an antimicrobial target; use of an antagonist of the protein for the manufacture of a medicament.

2. Claims: 2 and partially 1, 4-31

P. aeruginosa chitinase having N-terminal sequence II, III or IV, fragments and antibodies thereof; antigenic composition comprising the same; uses thereof for the detection, diagnostic, prophylaxis and treatmentof P. aeruginosa; nucleic acid molecule encoding the chitinase, derivatives and analogs thereof; vector and host cell comprising the same and use thereof in a vaccine composition and for the detection/diagnosis of P. aeruginosa; test for determining if the chitinase or fragments represent an antimicrobial target; use of an antagonist of the chitinase for the manufacture of a medicament.

INTERNATIONAL SEARCH REPORT

Information on patent family members

National Application No
PCT/GB 00/02554

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 543091 A	26-05-1993	DE 4128454 A	04-03-1993
		JP 6181793 A	05-07-1994
JP 10066568 A	10-03-1998	JP 3002140 B	24-01-2000
US 5114712 A	19-05-1992	NONE	
WO 9832769 A	30-07-1998	AU 5771798 A	18-08-1998
		CN 1244203 T	09-02-2000
		EP 0980389 A	23-02-2000

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